AD_____

GRANT NUMBER DAMD17-94-J-4339

TITLE: A Genetic Approach to Identifying Signal Transduction Mechanisms Initiated by Receptors for TGF-B-Related Factors

PRINCIPAL INVESTIGATOR: F. Michael Hoffman, Ph.D.

CONTRACTING ORGANIZATION: University of Wisconsin
Madison, Wisconsin 53706-4880

REPORT DATE: October 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | October 1998 | Final (1 Oct 94 - 30 Sep 98) |

**4. TITLE AND SUBTITLE** A Genetic Approach to Identify Signal Transduction Mechanisms Initiated by Receptors for TGF-Beta-Related Factors

**5. FUNDING NUMBERS**

DAMD17-94-J-4339

**6. AUTHOR(S)**

F. Michael Hoffman, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

University of Wisconsin
Madison, Wisconsin 53706-4880

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

**10. SPONSORING/MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**



**12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200*

*decapentaplegic* (*dpp*) is a Transforming Growth Factor beta (TGF-β)-related growth factor that controls multiple developmental processes in *Drosophila*. To identify components involved in *dpp* signaling, a modifier screen was carried out for dominant enhancer mutations of a hypomorphic allele of *thick veins* (*tkv*), a type I receptor for *dpp*. New alleles of *tkv, punt, Mothers Against dpp* (*Mad*) and *Medea* (*Med*), all of which are known to mediate *dpp* signaling, were recovered. Mutations in the *60A* gene, which encodes another TGF-β-related factor in *Drosophila*, were also recovered. The molecular identities of the new alleles of *60A* , *Mad* and *punt* were established by DNA sequence analysis. These results also provide the first *in vivo* evidence for the involvement of *60A* in the *dpp* pathway. We propose that *60A* activity is required to maintain optimal signaling by the *dpp* pathway, possibly by forming biologically active heterodimers with *dpp*. The enhancer mutations *D1* and *D2* potentially represent two new loci involved in *dpp* signaling. The *dpp*-like phenotypes caused by the enhancer mutations and their genetic interactions with members of the *dpp* pathway suggest that they are likely to be essential components in the *dpp* signal transduction pathway.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| Transforming Growth Factor, Signal Transduction, Genetic Approaches, Growth Inhibition, TGF-Beta, TGF-Beta Receptors, Genetic Strategies, Drosophila, Breast Cancer | 74 |
| | 16. PR[illegible] |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

X Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

NA For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

NA In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

D. Michael Huff 11/4/98

PI - Signature Date

**TABLE OF CONTENTS**

## INTRODUCTION

The transforming growth factor beta (TGF-β) superfamily consists of a growing number of identified growth factors regulating remarkably diverse aspects of cell life, including cell growth, differentiation, death, adhesion and migration. Since the first discovery of mammalian TGF-β1 16 years ago as a factor capable of inducing anchorage-independent growth of normal rat kidney fibroblasts (Roberts *et al.*, 1981), close to 40 TGF-β-related factors have been identified in organisms as diverse as worms, insects and human. Based on structural and functional relationships, members of this superfamily are subdivided into the prototypic TGF-β family (Massagué, 1990), the Activin/Inhibin family (Ying, 1989), the Bone Morphogenetic Protein (BMP) family (Hogan, 1996b) and other more distantly related factors including the Müllerian Inhibiting Substance (MIS) (Cate *et al.*, 1986), the Growth and Differentiation Factors (GDF) 1, 3, 9 (Lee, 1991; McPherron and Lee, 1993) and the Glial-Derived Neurotrophic Factor (GDNF) (Lin *et al.*, 1993). The BMPs constitute the largest subgroup, including mammalian BMPs 2-13, Nodal and GDF-5; chicken dorsalin 1; *Xenopus* Vgr-1 and Nodal related XNr1-3; and *Drosophila decapentaplegic* (*dpp*), *60A and screw* (*scw*) (Hogan, 1996b).

Even though TGF-βs were initially identified by their ability to transform certain cell lines, they are best known for their potent antiproliferative effects on cells from various lineages (Moses *et al.*, 1990; Massagué, 1990). Recent studies revealed that TGF-β partly acts through transcriptional activation of genes whose products directly interfere with cell cycle progression. TGF-β treatment increases the protein levels of p15, p21 and p27, members of the families of cyclin-dependent kinase (cdk) inhibitors (Sherr and Roberts, 1995). These inhibitors inactivate G1 cdks, resulting in hypophosphorylation of the retinoblastoma gene product, Rb and subsequent cell cycle arrest at the G1 to S transition (Reynisdottir *et al.*, 1995; Hannon and Beach, 1994; Datto *et al.*, 1995). The antiproliferative effect of TGF-βs is also mediated by rapid inhibition of genes that participate in G1 progression, such as members of the Myc family (Alexandrow and Moses, 1995). The observations that various tumor-derived cell lines are unresponsive to TGF-β (Fynan and Reiss, 1993) and that in certain epidermal tumors, the malignancy is associated with defects in autocrine TGF-β production (Glick *et al.*, 1994) suggest that TGF-β signaling has tumor-suppressing functions.

In addition to regulating cell growth, TGF-β-related factors, BMPs in particular, also play pivotal roles in development. The founding members of the BMP family were first identified based on their ability to stimulate ectopic bone formation when implanted in experimental animals (Wozney *et al.*, 1988). In fact, BMPs regulate various developmental processes by acting as morphogens to pattern cellular fields in a concentration dependent manner, as inducers of cell fates across germ layers and as messengers for the so-called community effect that coordinates

the differentiation of groups of cells (Moses and Serra, 1996; Hogan *et al.*, 1994; Hogan, 1996a; Hogan, 1996b). For example, in *Xenopus* embryonic tissues, cells express different genes depending on their distance from a source of activin, suggesting the operation of a morphogen gradient in vertebrates (Gurdon *et al.*, 1994). Similar concentration-dependent patterning of the *Xenopus* gastrula ectoderm by BMP-4 has also been demonstrated (Wilson *et al.*, 1997). In the chick limb, ectopic expression of BMP-2 in the anterior mesenchyme induces FGF-4 expression in the overlying apical ectodermal ridge (AER) (Duprez *et al.*, 1996). In mice, BMP-2, -4, and -7 are all expressed in the mesenchyme as well as in the AER of the developing limb bud, where they may signal to pattern the underlying mesenchymal progress zone (Hogan, 1996b). Similar cross tissue interactions mediated by BMPs are also important for mammalian tooth morphogenesis (Vaahtokari *et al.*, 1996). Recently, several spontaneous mutations were shown to affect murine BMP genes, including BMP-5 (*short ear*), GDF-5 (*brachypodism*) and *nodal*. In addition, five more BMP genes have been inactivated by targeted mutagenesis, including BMP-2, ,-4 ,-7 and -8B. Analysis of homozygous phenotypes has provided genetic evidence for the importance of BMPs in the establishment of the basic embryonic body plan and the development of nearly all organs and tissues (see references in Hogan, 1996b).

In a signaling system involving multiple interacting signaling components, a great diversity of cellular responses can be achieved through combinatorial actions such as the formation of heterodimers between distinct ligand monomers at sites of overlapping expression. One example of how the composition of a TGF-β-like molecule can drastically alter its activity is provided by the inhibins and activins, which share common subunits. Activins that are homodimers of β subunits have opposite biological activities to the inhibins, which are heterodimers of α and β subunits (Ling *et al.*, 1986; Petraglia *et al.*, 1989). In addition to having antagonistic functions, heterodimers can also function synergistically with the homodimers. For example, heterodimers of *Xenopus* BMP-4 and -7 were shown to be more potent in bone and mesoderm formation assays than either of the homodimers (Aono *et al.*, 1995; Suzuki *et al.*, 1997). In mice, the overlapping spatial expression of several BMPs suggests that these growth factors may combinatorially regulate cell fate specification (Lyons *et al.*, 1995).

The biosynthesis and maturation processes of TGF-β-related factors are tightly regulated and, to some extent, contribute to their versatile functions. These factors are synthesized as large precursor molecules that undergo proteolytic cleavages at a cluster of dibasic residues to yield mature bioactive carboxy termini (Roberts and Sporn, 1990; Panganiban *et al.*, 1990a; Doctor *et al.*, 1992). Most members of this family have seven conserved cysteines in the carboxy ligand domain. Structural analysis of TGF-β2 (Daopin *et al.*, 1992) and BMP-7 (Griffith *et al.*, 1996) revealed that six of the cysteines form a cysteine knot through intramolecular disulfide bonds and that the seventh cysteine participates in an intermolecular disulfide bond to form a dimer. Dimerizing with different partners can drastically alter the ligands'

biological activity, as best exemplified by the activins and inhibins. Activins, which are homodimers of β subunits have the opposite effect on the regulation of follicle stimulating hormone release than inhibins, which are heterodimers of α and β subunits (Ying, 1989). Upon secretion, the access of these growth factors to cell surface receptors is regulated by their complex interactions with various extracellular proteins (Miyazono *et al.*, 1993). For example, the binding of activin by follistatin, a soluble protein in ovary, prevents access of activin to its receptors (Hemmati-Brivanlou *et al.*, 1994). The binding of TGF-β2 to betaglycan, a membrane proteoglycan, profoundly increases its affinity for the signaling receptors (Lopez *et al.*, 1993). However, when expressed in a soluble form or released from the cell surface by the action of an endogenous protease, betaglycan sequesters TGF-β and acts as a potent antagonist of TGF-β binding to cell surface receptors (Lopez *et al.*, 1994).

Cross-linking experiments using radiolabeled ligands and expression cloning approaches have identified a novel class of transmembrane serine/threonine kinases as the major cell surface receptors for TGF-β-related growth factors (reviewed by Attisano *et al.*, 1994; Massagué and Weis-Garcia, 1996). These receptors are divided into the type I and type II families based on their distinct primary sequences and functions. Studies of the receptor binding profile of a panel of mutant epithelial cell lines resistant to TGF-β-induced growth arrest suggest that both types of receptors are required for signaling (Boyd and Massagué, 1989; Laiho *et al.*, 1990; Laiho *et al.*, 1991). Further biochemical and functional analyses of these cell lines have established the mechanism of receptor activation for TGF-β (Wrana *et al.*, 1994). The type II receptor is the principle ligand binding component and has a constitutively active kinase activity. Upon ligand binding by the type II receptor, the type I receptor is recruited to form a heteromeric complex with the type II receptor, which activates the type I receptor by phosphorylating it in a conserved cytoplasmic domain rich in glycine and serine (the GS domain). This mechanism places the type I receptor downstream of the type II receptor during signal transduction. Numerous studies have confirmed the generality of this mechanism. Some variation resides in the ligand binding properties of BMP receptors, in which case high affinity ligand binding requires cooperation between both types of receptors (see references in Attisano and Wrana, 1996)

The presence of multiple ligands, receptors and heteromeric receptor complexes highlights the combinatorial nature of TGF-β-related signaling. There is evidence that different downstream responses can be specified by distinct signal transducers. For example, ActRII and ActRIIB, two mammalian activin type II receptors, are also capable of binding BMP-7 under physiological conditions and of transducing activin-like effects (Yamashita *et al.*, 1995). Meanwhile, the recruitment of distinct activin type I receptors, ActRI or ActRIB into a complex with ActRIIB elicits different cellular responses in the presence of activin, suggesting that type I receptors play a central role in specifying downstream effects (Yamashita *et al.*, 1995).

This function of the type I receptor is likely linked to its kinase activity since type I receptors with highly related kinase domains transduce similar cellular responses even though the ligand and the type II receptor partners are distinct (Carcamo *et al.*, 1994).

Since the identification of the receptors for TGF-β-related growth factors, much effort has been focused on identifying components essential for relaying the signal from cell surface to nucleus. Several groups have used the yeast two hybrid system (Fields and Song, 1989) to look for proteins that directly interact with the cytoplasmic domains of TGF-β receptors. The TGF-β type I receptor (TβRI) interacting proteins identified include FK506/rapamycin binding protein (FKBP12) (Wang *et al.*, 1994) and farnesyl transferase-α (FT-α) (Kawabata *et al.*, 1995; Wang *et al.*, 1996a; Ventura *et al.*, 1996). TGFβ-receptor interacting protein-1 (TRIP-1) (Chen *et al.*, 1995) and apolipoprotein/clusterin (Reddy *et al.*, 1996) were identified based on their interactions with the TGF-β type II receptor (TβRII). FKBP12 has been postulated to function as a negative regulator of the TGF-β pathway based on the observation that blocking its interaction with TβRI using non-functional derivatives of FK506 leads to an enhancement of TGF-β signaling (Wang *et al.*, 1996b). Since mutations in TβRI that disrupt FKBP12 binding do not affect the signaling capacity of the receptor (Charng *et al.*, 1996), FKBP12 is more likely a modulator rather than a direct signal transducer of the pathway. The physiological relevance of other interacting proteins mentioned above in TGF-β signaling remains uncertain. TAK1 (TGF-β-activated kinase) was identified in a screen for members of the mammalian mitogen-activated protein kinase kinase kinase family (MAPKKK) capable of activating a yeast pheromone-induced MAPK pathway (Yamaguchi *et al.*, 1995). The activation of TAK1 leads to a typical TGF-β mediated transcriptional response and requires the kinase activity of TAK1 (Yamaguchi *et al.*, 1995). Subsequently, a yeast two hybrid screen identified TAB1, a novel protein which interacts with TAK1 and enhances its kinase activity (Shibuya *et al.*, 1996). These data suggest that TAK1 and TAB1 may mediate TGF-β signaling, but since the kinase activity of TAK1 is stimulated by TGF-β as well as BMP-4 (Yamaguchi *et al.*, 1995), their precise role in TGF-β signaling requires further investigation.

In *Drosophila*, a screen for maternal enhancer mutations of a hypomorphic *dpp* allele allowed identification of *Mothers against dpp* (*Mad*) and *Medea* (*Med*), which are required in many *dpp*-dependent developmental processes (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995). *Mad* is required in *dpp* responding cells (Newfeld *et al.*, 1996), suggesting that *Mad* plays a role in mediating *dpp* signaling. Mutations in *Mad* suppress dominant phenotypes generated by a constitutively active type I *dpp* receptor, placing *Mad* downstream of the receptors (Hoodless *et al.*, 1996; Wiersdorff *et al.*, 1996; Newfeld *et al.*, 1997). In *C. elegans*, attempts to recover mutations affecting dauer larvae formation, a process controlled by BMP-like factors, identified *sma-2*, *-3*, and *-4*, which encode proteins homologous to Mad (Savage *et al.*, 1996).

*Mad* homologues were soon identified in frog, mouse and human. These Mad-related proteins constitute the Smad family. Extensive biochemical and genetic analysis revealed that Smads are key cytoplasmic signal transducers in TGF-β-related pathways, linking receptor activation at the cell surface and target gene expression changes in the nucleus (reviewed by Derynck and Zhang, 1996; Massagué, 1996; Wrana and Attisano, 1996; Wrana and Pawson, 1997). Vertebrate Smad1 through 7 fall into three functionally distinct categories. Smad1, 2, 3 and probably Smad5 are receptor-regulated-Smads which associate directly with, and are phosphorylated within a conserved C terminal SSXS domain by, activated type I receptors (Hoodless *et al.*, 1996; Kretzschmar *et al.*, 1997; Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). The phosphorylated Smads then form a complex with Smad4 and accumulate in the nucleus (Lagna *et al.*, 1996; Macias-Silva *et al.*, 1996; Hoodless *et al.*, 1996; Zhang *et al.*, 1997; Wu *et al.*, 1997) where they can function as transcriptional activators. In *Drosophila*, Mad can directly bind the enhancer of *vestigial* and mediate its activation by *dpp* (Kim *et al.*, 1997). In *Xenopus*, Smad2 interacts with the fork-head-DNA-binding protein, FAST1, to activate the activin responsive element in the mix.2 gene (Chen *et al.*, 1996). The receptor-regulated-Smads seem to specify the biological responses in that Smad1 and 5 function in BMP signaling (Hoodless *et al.*, 1996; Kretzschmar *et al.*, 1997; Liu *et al.*, 1996; Thomsen, 1996) while Smad2 and 3 function in TGF-β and activin signaling (Baker and Harland, 1996; Eppert *et al.*, 1996; Graff *et al.*, 1996; Lechleider *et al.*, 1996; Macias-Silva *et al.*, 1996; Yingling *et al.*, 1996). Structural and mutational analysis of the conserved C terminal domain of Smad4 suggests that Smads form homotrimers and that the phosphorylated receptor-activated-Smads may undergo a conformational change which enables them to form a heterohexamer with the homotrimeric Smad4 protein (Hata *et al.*, 1997; Shi *et al.*, 1997). More recently, a new class of Smads including mammalian Smad6 and 7, the *Drosophila Daughters against dpp* (*Dad*) and the *C. elegans* Daf-3 have been found to function as antagonists in TGF-β-related signaling (Hayashi *et al.*, 1997; Nakao *et al.*, 1997; Imamura *et al.*, 1997; Tsuneizumi *et al.*, 1997; Patterson *et al.*, 1997). Thus, Smad proteins can both positively and negatively regulate TGF-β-related signaling. Consistent with the notion that the TGF-β pathway has a tumor-suppressing function, mutations in Smads are associated with various types of tumors (Hahn *et al.*, 1996; Schutte *et al.*, 1996; Eppert *et al.*, 1996; Thiagalingam *et al.*, 1996).

Given the remarkably diverse physiological functions of the TGF-β-related factors, understanding their mode of action should provide new strategies to treat cancer, to repair damaged tissues and to modulate development. However, the vast number of TGF-β-related factors in vertebrates and the potential interactions and functional redundancy among related factors make assigning precise roles to each factor a daunting task. The identification of TGF-β-related pathways in *Drosophila* enabled us to take advantage of the power of genetics in this relatively simple organism to begin addressing some of the fundamental questions regarding TGF-β-related signal transduction.

In *Drosophila*, the TGF-β-related factors are represented by the *decapentaplegic* (*dpp*), *60A* and *screw* (*scw*) gene products (Padgett *et al.*, 1987; Doctor *et al.*, 1992; Wharton *et al.*, 1991; Arora *et al.*, 1994). All three are members of the BMP family. At the amino acid level, *dpp* is 75% identical to mammalian BMP-2 and -4 and 60A is 70% identical to BMP-5 through -8 (Kingsley, 1994a). When implanted subcutaneously in rats, Dpp and 60A proteins are able to induce ectopic bone formation (Sampath *et al.*, 1993). Furthermore, human BMP-4 is able to rescue the dorsal/ventral patterning defects of *dpp* mutant embryos (Padgett *et al.*, 1993), demonstrating the functional conservation of the dpp/BMP-4 pathway throughout evolution. Scw proteins have been proposed to enhance *dpp* activity during early embryogenesis by forming heterodimers with Dpp (Arora *et al.*, 1994). The role of *60A* in development has remained unclear due to the lack of mutations in the *60A* gene.

*dpp* has a dynamic expression pattern correlating with its multiple functions throughout *Drosophila* development. At the blastoderm stage, *dpp* transcripts are restricted dorsally (St. Johnston and Gelbart, 1987) where *dpp* functions as a morphogen to specify distinct dorsal structures (Ferguson and Anderson, 1992b; 1992a; Wharton *et al.*, 1993). Later during embryogenesis, *dpp* is expressed in the ectoderm (Jackson and Hoffmann, 1994), where it induces differentiation of the dorsal mesoderm (Staehling-Hampton *et al.*, 1994). In the visceral mesoderm, *dpp* is expressed in discrete domains to regulate the expression of several homeotic genes and to induce the expression of the homeotic gene *labial* (*lab*) in the underlying endoderm (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990b) . *dpp* is also expressed in specific positions in the larval imaginal discs (Masucci *et al.*, 1990) to control the proliferation and patterning of adult appendages (reviewed by Brook *et al.*, 1996).

In *Drosophila*, two type I receptors encoded by *saxophone* (*sax*) and *thick veins* (*tkv*) and one type II receptor encoded by the *punt* gene have been shown to be functional *dpp* receptors (Penton *et al.*, 1994; Brummel *et al.*, 1994; Nellen *et al.*, 1994; Ruberte *et al.*, 1995; Letsou *et al.*, 1995; Xie *et al.*, 1994). *Mothers against dpp* (*Mad*) and *schnurri* (*shn*) are two factors identified through genetic interactions with *dpp* (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995; Staehling-Hampton *et al.*, 1995; Grieder *et al.*, 1995; Arora *et al.*, 1995). *shn* encodes a protein related to human zinc finger transcription factor PRDII/MBPI/HIV-EP1 (Staehling-Hampton *et al.*, 1995; Arora *et al.*, 1995; Grieder *et al.*, 1995). Mad-related proteins (Smads) have been isolated from a wide range of distantly related organisms. Genetic and biochemical evidence indicates that they are key signal transducers, linking events between receptor activation and changes in target gene expression (Derynck and Zhang, 1996; Massagué, 1996; Wrana and Pawson, 1997).

The rapid advance made in understanding the mechanisms by which TGF-β-related signals are relayed from cell surface to nucleus clearly demonstrates the power of genetic approaches to dissecting a biological pathway. Modifier genetics

utilizes the synergistic interactions between components in the same biological pathway. In such a system, if the signaling output is just below the optimal level and produces a visible phenotype, then the pathway is likely to be sensitive to a reduction in the levels of other essential components in the same pathway, resulting in a enhanced or suppressed phenotype, depending on whether the component is a positive or negative regulator of the pathway. In *Drosophila*, modifier genetics has been successfully used to isolate essential components in the *sevenless* receptor tyrosine kinase pathway (reviewed by Hafen *et al.*, 1993) and in the cytoplasmic *Abelson* tyrosine kinase pathway (reviewed by Hoffmann, 1991). In both cases, the characterization of second-site modifiers has been instrumental in understanding how similar pathways may function in higher organisms.

The dosage sensitive nature of the *dpp* signaling pathway prompted us to use modifier genetics to isolate new components in the pathway. Using the phenotypes generated by a hypomorphic *dpp* receptor as a genetic background, we screened for enhancer mutations that exacerbate the phenotype. New alleles of many genes known to be involved in the *dpp* pathway were recovered, as well as mutations in *60A*, a BMP-7 homologue, whose function was not previously known.

## EXPERIMENTAL METHODS

### Source of antibodies

Anti-Scr, anti-Ubx, anti-Lab antibodies were gifts of Dr. Matthew Scott, Stanford University. Anti-Wg antibody was a gift of Dr. Roel Nusse, Stanford University. Anti-Dpp antibody was described in Panganiban *et al.* (1990a). The mouse monoclonal anti-En antibody mAb 4F11 were described in Patel *et al* (1989). The rabbit anti-β-galactosidase antibodies were purchased from Rockland. The mouse anti-β-galactosidase antibodies were purchased from Promega. The biotin conjugated secondary antibodies, the horse radish peroxidase (HRP) conjugated secondary antibodies and the HRP conjugated streptavidin were purchased either from Vector Laboratories Inc., Boehringer Mannheim, GIBCO BRL or Jackson ImmunoResearch.

### Anti-Dpp staining

Anti-Dpp staining was done according to the procedures described in Panganiban *et al.* (1990b) with minor modifications. Briefly, staged embryos were dechorionated in 50% bleach and were then fixed in 1 part heptane and 1 part 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes. The fixed embryos were devitellinized in 1 part heptane and 1 part methanol, followed by rinses in methanol. The embryos were used immediately for staining. After rehydration in PBS, the embryos were blocked in PBSTB (PBS with 0.1% Triton X-100 and 0.2% bovine serum albumin (BSA)) and 5% normal goat serum (NGS) for at least 3 hours. The embryos were then incubated with the pre-absorbed rabbit anti-Dpp antibodies (1 μg/ml) in PBSTB with 5% NGS at 4°C overnight. The stained embryos were washed for at least 1 hour at room temperature in PBSTB with at least four changes of wash solutions and blocked for half an hour in PBSTB with 5% NGS. Embryos were then incubated with biotinylated anti-rabbit-IgG secondary antibodies (1:300) in PBSTB with 5% NGS at room temperature for at least one hour. After washing with PBSTB as described for the primary antibody incubation, the embryos were incubated with HRP-conjugated streptavidin (1:300) in PBSTB plus 5% NGS for at least one hour at room temperature. The embryos were washed as described above. The peroxidase reactions were done in 1X diaminobenzidine (DAB) solution (0.25 mg/ml in 100 mM Tris, PH 7.5) with 0.003% $H_2O_2$ and 0.08% of $CoCl_2$ ad $NiCl_2$. The reaction was monitored under a dissecting scope and stopped by washing with PBSTB when the background appeared lightly stained. After thorough washing, the stained embryos were dehydrated in an ethanol series and mounted in 80% glycerol. The embryos were visualized with Nomarski optics and pictures were taken with Kodak Royal Gold 25 film.

### Anti-Lab, anti-Wg, anti-Scr, anti-Ubx and anti-Antp stainings

Anti-Lab (1:200), anti-Wg (1:300), anti-Scr (1:10), anti-Ubx (1:5) and anti-Antp (1:5) stainings were essentially the same as anti-Dpp staining except that the embryos were fixed in 1 part of heptane and 1 part 4% formaldehyde in PMG buffer ( 0.1 M PIPES, 2 mM EGTA and 1 mM $MgSO_4$). For anti-Wg staining, lowering the NGS

concentration from 5% to 1% gave better staining results. For anti-Lab and anti-Wg stainings, a biotin conjugated secondary antibody and an HRP conjugated streptavidin were used. For anti-Scr, anti-Ubx and anti-Antp stainings, an HRP conjugated anti-mouse IgG antibody was used.

**Anti-β-galactosidase double-staining**

Sequential staining reactions were done for embryos needed to be double-stained with the anti-β-galactosidase antibody. Briefly, the embryos stained with the first antibodies were developed in DAB solution containing nickel and cobalt ions as described above. The stained embryos were washed extensively and blocked as described and then incubated with either rabbit anti-β-galactosidase antibodies (1:800) or mouse anti-β-galactosidase antibodies (1:300). After incubation with appropriate HRP conjugated secondary antibodies, the anti-β-galactosidase antibodies were detected by the oxidation of DAB in the absence of nickel and cobalt ions.

**Anti-Engrailed and anti -β-galactosidase stainings**

Imaginal discs were dissected in PBS and fixed in 2% formaldehyde in PIPES buffer (0.1 M PIPES (PH 6.9), 2 mM $MgSO_4$, 1 mM EGTA, 1% NP-40) for 20 minutes, washed in the wash buffer (PBS with 0.3% Triton X-100 (PT) and 1 mg/ml BSA) and blocked in PTB buffer (PT with 5 mg/ml BSA) for 1 hour. Mouse monoclonal antibody, mAb 4F11 for Engrailed protein (Patel *et al.*, 1989) (1:25 dilution) and rabbit anti-βgalactosidase (1:800 dilution, Rockland) for *wg-lacZ* were used in PTB buffer at 4°C overnight. The discs were washed in the wash buffer for at least one hour with frequent changes. Secondary antibodies used were FITC conjugated anti-mouse IgG and rhodamine conjugated anti-rabbit IgG (both at 1:200 dilution, Jackson ImmunoResearch). After washes, the stained discs were mounted in the Glycerol Mount (30% glycerol, 5 mM Tris (PH 8.8), 150 mM NaCl, 0.02% $NaN_3$). To prevent photo bleaching, PDA (p-Phenylenedianime P1519, Sigma) was added to the Glycerol Mount at a final concentration of 0.5 mg/ml). The images were collected using the confocal microscope at the W. M. Keck Foundation neural imaging laboratory.

***Drosophila* stocks**

*Drosophila* stocks were cultured on standard cornmeal yeast extract and sucrose medium at 25°C, unless otherwise specified. *Canton S.* was used as the wild type stock. $sax^5$ was described in Twombly *et al* (1996). $Mad^P$ is described in Sekelsky *et al* (1995). $Med^4$ was described in Raftery *et al* (1995). $p[w^+$UAS-$tkv^{QD}]$ was described in Nellen *et al* (1996). The *vestigial*-$Gal4^{181}$ line has a *vestigial* intron 2 enhancer (Williams *et al.*, 1994) that drives the expression of Gal4 proteins in the anterior and posterior hinge regions of the wing disc (Morimura and Hoffmann, unpublished). All other mutants and chromosomes are referenced or described in Linsley and Zimm (1992). Mutant stocks were obtained from

Bloomington, Umea stock centers, Berkeley *Drosophila* genome project and from A. Letsou, V. Twombly, M. Singer, W. Gelbart and K. Wharton.

**N-ethyl-N-nitrosourea (ENU) mutagenesis**

One gram of ENU (Sigma, ISOPAC) was dissolved in 10 ml of 95% ethanol and diluted with 90 ml of the phosphate/citrate buffer (1 M $Na_2HPO_4$, 0.05 M $Na_3C_6H_5O_7$, PH 5.0, adjusted with phosphoric acid). An appropriate amount of the stock ENU solution (10 mg/ml) was further diluted with 1% sucrose solution to give a final concentration of 0.4 mg/ml (3.4 mM). All manipulations were performed in a fume hood and the ENU solution was made fresh. 1M NaOH solution was used to soak the containers and the waste overnight to neutralize the ENU.

Initial attempts to recover modifiers of $tkv^6$ in an F1 screen were not successful because of greatly reduced viability and fertility of the flies with enhanced phenotypes. Thus an F2 screen was carried out. *pr cn* was recombined onto the $tkv^6$ chromosome and the stock was made to be isogenic for the second and third chromosomes. Young $tkv^6$ *pr cn/CyO* males were collected and aged at 25°C for 5 days or at room temperature for 7 days. The males were starved for at least 12 hours prior to feeding 3.4 mM ENU in 1% sucrose solution for 18 to 24 hours. The mutagenized males were mated to females that have a reciprocal translocation between *CyO* and *TM6,B (T(2;3))* to force the cosegregation of the second and third chromosome. The males were removed after three days of mating to prevent clustering effect. Individual male progeny were mated to $tkv^6$*/CyO; TM2/TM6,B* females. Enhancement of the imaginal disc derived structures were screened in the progeny homozygous for $tkv^6$. The chromosomes with the enhancer mutations were recovered from the siblings of the enhanced progeny and crossed to $tkv^6$*/CyO; TM2/TM6,B* females for retesting and to establish balanced stocks.

The number of complementation groups were determined from *inter se* crosses among enhancers. For enhancers on the second chromosome, a *tkv* transgene on the third chromosome, *P[w+tkv]1C-10* was used to compensate for the $tkv^6$ mutation. Allelism with known mutations was established by genetic non-complementation and by meiotic and deletion mapping. The *Sp Bl* $L^{rm}$ *Bc* $Pu^2$ $Pin^B$ chromosome was used for meiotic mapping of the enhancers on the second chromosome. The *ru h th st cu sr e ca* chromosome was used to map the enhancers on the third chromosome. The "deletion kit" from Bloomington Stock center (Indiana) was used for deletion mapping.

**Gamma ray mutagenesis**

Non-isogenized *Canton S.* males were used. The males were aged as described for the ENU mutagenesis and exposed to 4,500 rads of γ-rays from a $^{137}Cs$ source (Mark I Irradiator). The mutagenized males were allowed to mate to

*In(2LR)Gla Bc/CyO* females for 3 days. Individual *Cy* male progeny from this cross were mated to either *tkv*$^6$ *pr cn D1/CyO*, *tkv*$^6$ *pr cn D2/CyO* or *tkv*$^6$ *pr cn D4/CyO* females. The crosses were scored for the absence of *Cy* progeny. The lethal mutations were recovered from siblings and crossed to the appropriate tester stocks for retesting and to establish balanced stocks.

**P element mutagenesis**

*w; P[w*$^+$*]l(2)k13214/CyO* (Berkeley *Drosophila* genome Project) females were mated to *w; Sp/CyO; Sb Δ2-3/+* males. w; *P[w*$^+$*]l(2)k13214/CyO; Sb Δ2-3/+* male progeny were then crossed to w; In(2LR)Gla Bc/CyO females. Individual *w*$^+$, *Sb*$^+$ and *Cy* male progeny were mated to *w; tkv*$^6$ *pr cn D4/CyO; TM2/TM6,B* females. The crosses were screened for the absence of *Cy* progeny. The lethal mutations were recovered from the siblings and were mated to *w; tkv*$^6$ *pr cn D4/CyO; TM2/TM6,B* females for re-testing and to establish balanced stocks.

**Genetic Crosses**

*wg-lacZ* was recombined onto the *tkv*$^7$ chromosome. *w; tkv*$^7$ *wg-lacZ/Df(2L) tkv2; P[w*$^+$*; tkv] 1A-8/TM2* adult flies were generated by mating *w; tkv*$^7$*wg-lacZ/In(2LR) Gla Bc; P[w*$^+$*; tkv] 1A-8/TM2* females to *Df(2L) tkv2/In(2LR) Gla, Bc; P[w*$^+$*; tkv] 1A-8/TM2* males. The *w; tkv*$^7$ *wg-lacZ/Df(2L) tkv2; P[w*$^+$*; tkv] 1A-8/TM2* larvae were identified by the absence of the *Bc* marker.

For enhancement mapping of the enhancers on the second chromosome, *tkv*$^6$ *pr cn */CyO* (asterisk indicates the enhancer mutation) males were crossed to *Sp Bl L*$^{rm}$ *Bc Pu*$^2$ *Pin*$^B$ /CyO females. The resulting *Cy*$^+$ female progeny were crossed to *In(2LR)Gla Bc/CyO* males. Males that were *Sp*$^+$ and balanced over *CyO* were collected to maximize the chances of retaining the *tkv*$^6$ mutation and individually crossed to *tkv*$^6$*/CyO; TM2/TM6,B* females. The *Cy*$^+$ progeny were scored for dominant markers and the presence or absence of enhancement effects.

For mapping the lethality of *D2* over the γ-ray allele *D2-1*, *tkv*$^6$ *pr cn D2/CyO* males were crossed to *Canton S.* females. The resulting *Cy*$^+$ females were crossed to *In(2LR)Gla Bc/CyO* males. Preliminary meiotic mapping based on enhancement of *tkv*$^6$ indicated that *D2* is between *Bl* and *L*$^{rm}$, so two nearby markers, *pr* and *cn* were used for the lethality mapping. Using the *pr cn* markers present on the *CyO* chromosome, the *Cy* male progeny were grouped by genotype with regard to the *pr* and *cn* loci (i. e. grouped by *pr*$^+$ *cn*$^+$; *pr cn*$^+$; *pr*$^+$ *cn* or *pr cn*). These males were then individually crossed to *D2-1/CyO* females and the crosses were scored for the presence or absence of *Cy* progeny. The distance between the *pr, cn* and the lethal locus was calculated by the recombination frequency observed.

Adult flies with the enhanced imaginal disc phenotypes were generated by mating *tkv*$^{6}$ *pr cn*/CyO* (asterisk indicates the enhancer mutations) males to *tkv*$^{6}$ *pr cn/CyO* females. The *tkv*$^{6}$*Mad*$^{D24}$/+ embryos were generated for cuticle preparations by mating *tkv*$^{6}$*Mad*$^{D24}$*/CyO* females to *Canton S.* males. The *w; tkv*$^{6}$ *pr cn Mad*$^{D3}$ or *tkv*$^{6}$ *pr cn D1*/Gal4-*vg*$^{181}$; P[*w*$^{+}$ UAS-*tkv*$^{QD}$]/+ adult flies were generated by mating *w;* Gal4-*vg*$^{181}$; + females to *w; tkv*$^{6}$ *pr cn Mad*$^{D3}$ or *tkv*$^{6}$ *pr cn D1/In(2LR)Gla Bc;* P[*w*$^{+}$ UAS-*tkv*$^{QD}$]*/TM2* males.

The *60A*$^{D4}$*/60A*$^{D8}$ larvae were generated by mating *60A*$^{D4}$*/CyO*-HS*hid* males to *60A*$^{D8}$*/CyO*-HS*hid* females. The cross was heat shocked on day 3 and day 4 for 2 hours at 37°C to kill progeny classes inheriting the *CyO*-HS*hid* chromosome. Escapers were observed when the heat shock was done after the early second instar stage. The *In(2LR)Gla Bc* chromosome was not used for identification of homozygous mutants because it does not suppress recombination on distal 2R where *60A* is located.

The embryos used for antibody staining were generated by mating the appropriate mutants balanced over a *CyO* chromosome marked with an *elav-lacZ* enhancer trap. After double staining with the antibodies of interest and anti-β-galactosidase antibody, the homozygous mutants were identified by the absence of anti-β-galactosidase staining.

**Preparation of larval gut, cuticle and adult appendages**

Larval guts were dissected and mounted according to Masucci and Hoffmann (1993). Briefly, third instar larvae were dissected and fixed in 50% ethanol for 5 to 10 minutes. Larval guts were then mounted in 90% glycerol and photographed using bright field optics. Cuticles were prepared as described previously (Struhl, 1989). Briefly, dechorionated, devitellinized embryos or first instar larvae were fixed in 1:4 glycerol: acetic acid for one hour at 60°C. The embryos were mounted in Hoyer's mounting media (Ashburner, 1989) and allowed to clear overnight at 60°C. Cuticles were photographed under phase contrast optics using Kodak Techpan high contrast film or Kodak Royal Gold 25 film. Wings and legs were mounted in Gary's magic mounting media (Ashburner, 1989) and photographed under bright field optics using Kodak Royal Gold 25 film.

**Molecular cloning procedures**

Unless otherwise described, the molecular cloning procedures were performed following standard protocols as described in Sambrook *et al* (1989).

**Cosmid library screening**

The probes used for the library screening were labeled with α-$^{32}$P-dCTP using the random priming method. Briefly, 20 to 100 ng of DNA was denatured with 2 μg of random nanomer primers in a boiling water bath for 5 minutes, followed by

incubation on ice for 2 minutes to allow annealing to occur. The annealed template DNA and primers were incubated with 50 mM dATP, dTTP, dGTP mix, 50 μCi of α-$^{32}$P-dCTP, 1X Klenow reaction Buffer and 2-3 units of Klenow enzyme in a total volume of 25 μl for 25 minutes at 37°C. The unincorporated dNTPs were removed using the NucTrap Probe Purification Columns (Stratagene). The probes were denatured in 0.2 N NaOH at room temperature for at least 10 minutes before use. Labeled probes were used immediately or stored at -20°C.

The iso-1 cosmid genomic library (gift of Dr. Tamkun, University of California, Santa Cruz) was plated onto LB/Amp (40 μg/ml) plates at a density of 3,000 to 5,000 colonies/150 mm plate. After overnight growth, the plates were chilled at 4°C for at least an hour to prevent colonies from streaking during lifting procedures. The colonies were lifted onto Amersham $N^+$ Hybond nylon membranes. The filters were then denatured for 5 minutes in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and neutralized for 5 minutes in the neutralizing solution (0.5 M Tris PH 7.5, 1.5 M NaCl). After neutralization, the filters were cross-linked using a Stratagene UV-Crosslinker on automatic setting. The cross-liked filters were washed in low stringency wash buffer (2XSSC, 1% SDS) for at least 1 hour at 65°C and pre-hybridized in the hybridization buffer (120 mM $Na_2HPO_4$, PH 7.2, 250 mM NaCl, 7% SDS, 10% PEG 8000, 30% formamide) for at 5 minutes at 37°C. Following pre-hybridization, the filters were incubated with fresh hybridization buffer and the labeled probes (1X$10^6$ CPM/ml) overnight at 37°C. After hybridization, the filters were first washed for half an hour in the low stringency wash buffer at 37°C with 2 changes, followed by washing for 2 hours in the high stringency buffer (0.1XSSC, 1% SDS) at 65°C with 4 changes. Autoradiography was done at -80°C with an intensifying screen.

A 5 kb RI rescued fragment from *60A*$^{D4\text{-}P2}$ genomic DNA was used as a probe to screen about 30,000 independent clones and four positive clones were isolated. Restriction enzyme digest and Southern analysis using internal fragments of the cosmid allowed the construction of the maps of the *tkv* and *60A* genomic region.

**Genomic Southern analysis**

Genomic DNA was isolated according to the method of Ashburner (1989). The DNA was digested to completion with appropriate restriction enzymes and separated on agarose gels. Gels were treated with 0.2 M HCl for 15 minutes followed by denaturation in 0.4 N NaOH for 45 minutes. The DNA was then transferred to the Zeta Probe nylon membranes (BioRad) in 0.4 N NaOH using a vacuum blotter (Pharmacia). The hybridization and washing conditions were essentially the same as described for library screenings.

**Plasmid rescue**

Twenty-five to 30 flies were homogenized in 700 μl of homogenization buffer (HB) (0.2 M sucrose, 0.1 M Tris, PH 9.2, 50 mM EDTA, 0.5% SDS) and incubated at 68°C for 10 minutes. One hundred and five μl of 8 M KOAc was added and the

homogenate was incubated on ice for 15 minutes. After spinning down the debris, the supernatant was collected and the DNA was precipitated for at least 1 hour at room temperature after adding one volume of isopropanol. The DNA was resuspended in TE and NaCl was added to a final concentration of 0.1 M. The DNA solution was extracted with phenol/chloroform and precipitated again by adding 2 volumes of ethanol. The genomic DNA was resuspended to a concentration of 1 fly equivalent/µl (FE). Five FE of genomic DNA was digested with the appropriate restriction enzymes in the presence of RNase (EcoRI was used to recover the genomic DNA on one end of the P insertion; BglII was used to recover genomic DNA on the other end of the insertion. For a map of the P-lacZW vector, see (Bier *et al.*, 1989)). The restriction enzyme reaction was phenol/chloroform extracted and the DNA was precipitated and briefly dried. The intramolecular ligation was set up in a 1 ml volume with 1 unit of the ligase (Boehringer Mannheim). Following incubation overnight at 16°C, the ligation reaction mixture was phenol/chloroform extracted and precipitated by adding 1 volume of PEG solution (1.6 M NaCl, 13% PEG 8000) and resuspended in 10 µl of dd$H_2O$. Two µl of the resuspended DNA was used for electroporating into DH5α *E. coli* using the Zapper Elctroporation Unit (UW Medical Electronics Lab) and transformed bacteria were plated onto LB/AMP (50 µg/ml) plates.

**Sequencing of mutant alleles**

Total RNA was isolated from heterozygous *Mad* and *punt* females and heterozygous *60A* males using the Tri Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. Gene specific cDNAs were reverse transcribed and amplified using the Superscript Preamplification System (GIBCOL BRL). A 3' gene specific primer was used to prime the synthesis of the first strand cDNA. The PCR reactions were performed with a Perkin Elmer 9600 thermal cycler under the following condition: 94°C for 2 minutes for hot start PCR, 30 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes, followed by 10 minutes at 72°C. In many cases, the PCR products were a mixture of specific and nonspecific products. The PCR products were shotgun subcloned into the TA cloning vector (Invitrogen) and colony lifts were prepared and screened using probes specific for the cDNA of interest. The positive clones were then sequenced on an automated sequencer (ABI 373). Sequencing multiple alleles of the same gene allowed identification of the polymorphisms specific to the mutagenized chromosomes. For $60A^{D4}$ and $60A^{D8}$, the mutant cDNAs for these two alleles were under represented (see Chapter 3, Table 3.5), so the genomic region of *60A* was sequenced. Briefly, 3 male flies were briefly homogenized with a toothpick in 50 µl of buffer containing 10 mM Tris (PH 8.2), 1 mM EDTA, 25 mM NaCl and 200 µg/ml of proteinase K. The mixture was incubated at 95°C for 15 minutes and after addition of 2.5 µl of 100 mM Pefabloc, for another 15 minutes at 65°C, then spun at 13,000Xg to remove the debris. Ten µl of the supernatant was used for PCR amplification under the same cycling conditions described above. The genomic PCR products were screened and sequenced in the same way as for the cDNAs.

## RESULTS

### *shn* and *punt* enhance $tkv^6$ phenotypes

Prior to conducting the modifier screen, different genetic backgrounds were evaluated. The $tkv^6$ mutation was chosen based on its genetic behavior. $tkv^6$ is a mutation in a splice acceptor site that results in aberrant in-frame splicing and deletes two amino acids in the extracellular domain of the type I receptor. When expressed in COS1 cells, the mutant receptor fails to bind BMP2 homodimers (Penton *et al.*, 1994). However, $tkv^6$ behaves genetically as a hypomorph with phenotypes much weaker than a null allele. It is homozygous viable and the only visible phenotype is thickened wing veins. All other imaginal disc derived structures from $tkv^6$ homozygotes appear normal. Interestingly, $tkv^6/Df(2L)tkv2$ flies are phenotypically identical to $tkv^6$ homozygotes. To test if $tkv^6$ was a suitable genetic background for a modifier screen, we examined the effects of lowering the activity of other known *dpp* pathway components. We found that heterozygous mutations in *shn* or *punt* enhanced the $tkv^6$ homozygous phenotype. In the $tkv^6$ background, $shn^{1B}$ was a dominant enhancer of the venation pattern in the wing and of proximal/distal patterning in the leg. In the wing, longitudinal vein 2 failed to reach the wing margin. In the leg, distal elements such as claws and distal tarsal segments were deleted. Such phenotypes were reminiscent of hypomorphic *dpp* mutant phenotypes (Spencer *et al.*, 1982). $punt^{135}$ was also an enhancer of the $tkv^6$ phenotypes. Based on these observations, we reasoned that the *dpp* signaling output through the mutant receptor $tkv^6$ was near the threshold for proper patterning of the imaginal discs. It was therefore an appropriate genetic background for identifying new components essential for mediating *dpp* signaling.

### Enhancers of *tkv* are phenotypically similar to *dpp* mutants

The F2 screen for enhancers of $tkv^6$ was conducted as outlined in Figure 1. The F2 progeny were screened for enhanced phenotypes in the imaginal disc derived structures. Over 10,000 mutagenized genomes were screened and fourteen dominant enhancers defining seven loci were recovered (Table 1). All of the enhancers were recessive lethal in a wild type background. $tkv^6$ homozygotes that are heterozygous for the enhancer mutations display defects in imaginal disc development with varying penetrance. During normal pupal development, the dorsal proximal region of the two wing imaginal discs fuse to form the adult notum. In $tkv^6$ flies, the notum appeared normal with a smooth contour and orderly oriented sensory bristles. This pattern was disrupted by the heterozygous enhancer mutations which resulted in a medial cleft in the notum, usually accompanied by abnormally parted bristles on both sides of the cleft. $tkv^6$ flies had normally patterned legs. However, a heterozygous *D4* mutation caused deletions of the distal and dorsal structures and occasional duplication of ventrolateral structures such as sex combs on male prothoracic legs. These phenotypes were indistinguishable from

those of $dpp^{disk}$ alleles (Spencer *et al.*, 1982), suggesting that these enhancers act in the *dpp* signal transduction pathway.

**Enhancer mutations show dosage sensitive interactions with various mutations in the *dpp* pathway**

Additional evidence that the enhancers mediate *dpp* signaling came from their dosage sensitive genetic interactions with other known mutations in the *dpp* pathway, including $dpp^{s5}$, *Df(2L)tkv2*, $sax^{5}$, $punt^{135}$, $Mad^{P}$ $Med^{4}$ and $shn^{IB}$ (Table 2). In many cases, the enhancers failed to fully complement these mutations and showed imaginal disc development defects ranging from gaps in wing veins to the notum and leg phenotypes. Since the enhancer mutations are induced in a $tkv^{6}$ background, all enhancer mutations on the second chromosome used in this test also have a $tkv^{6}$ mutation. $tkv^{6}$ alone showed no detectable heterozygous interactions with the *dpp* pathway mutations examined except for the thickened venation phenotype when it was in trans to $tkv^{6}$ or *Df(2R)tkv2*, indicating that the phenotypes observed were due to the presence of the enhancer mutations.

To test whether the maternally provided enhancer mutations interfere with early embryonic *dpp* signaling, we determined whether mutant alleles of the enhancer mutations in the mother would sensitize the zygote to the level of *dpp* function. The *dpp* locus is haploinsufficient, indicating that the level of *dpp* function in the early embryo is critical (Irish and Gelbart, 1987). A series of *dpp* mutant alleles have been identified that vary in their amount of residual activity (Wharton *et al.*, 1993). One such allele, $dpp^{hr27}$, normally provides enough residual function to allow viability as a heterozygote. We tested whether mothers heterozygous for $dpp^{hr27}$ and the enhancer mutations would shift the threshold requirement for *dpp* function, such that $dpp^{hr27}$ would become a dominant lethal in the zygote. In previous studies, certain alleles of *tkv, sax, Mad* and *Med* were show to possess this maternal enhancement property (Penton *et al.*, 1994; Nellen *et al.*, 1996; Raftery *et al.*, 1995). Of the enhancer mutations tested, maternally provided *D1, D14* and *D16* significantly reduced the zygotic viability of $dpp^{hr27}$ (Table 3). However, the lack of enhancement in this test does not necessarily rule out the involvement of the enhancer mutations in the *dpp* pathway, since in the cases of *tkv* and *sax*, dominant negative, rather than loss of function mutations, are required for enhancement (Penton *et al.*, 1994; Nellen *et al.*, 1994).

**Dominant enhancers of $tkv^{6}$: new alleles of *tkv, Mad, Med, punt* and *60A***

Meiotic mapping and complementation tests established seven complementation groups for the enhancers. As expected based on the initial evaluation of the $tkv^{6}$ genetic background, new alleles of *tkv, Mad, Med* and *punt* were recovered (Table 1). We sequenced the coding regions of the new *Mad* and *punt* alleles to determine their molecular lesions (Table 5 and Figure 2). Of the five *Mad* alleles, three had point mutations in the coding region (Figure 2). Missense

mutations were found in both new *punt* alleles (Figure 2). $tkv^{D17}$ and $Med^{D5}$ allelism was based on genetic non-complementation. In addition, a *tkv* transgene was able to rescue the lethality of $tkv^{D17}$ homozygotes, supporting the conclusion that *D17* is a *tkv* allele (data not shown).

Three of the new *Mad* alleles, $Mad^{D3}$, $Mad^{D15}$ and $Mad^{D24}$ display dominant female sterility. Embryos derived from heterozygous females had severe patterning defects. The majority of the embryos were partially ventralized. The head structures remained external and the head skeletons were partially deleted. The most posterior structures remained internalized. About 10% of the embryos failed to initiate the germ band retraction process during gastrulation, resulting in a "U" shaped morphology. The dominant female sterility of $Mad^{D3}$ and $Mad^{D15}$, but not of $Mad^{D24}$, can be reverted by addition of a *tkv* transgene or a *dpp* transgene maternally (data not shown).

**Preliminary characterizations of the *D1* and *D2* loci**

Since the *D1* and *D2* complementation groups were only represented by a single ENU allele each, gamma ray screens were carried out to recover more alleles of both loci according to the scheme outlined in Figure 3. No mutations allelic to the *D1* locus were recovered. The mutations, *D2-1*, *D2-2* and *D2-3*, failed to complement $tkv^{6}D2$. *D2-2* was determined to be most likely allelic to *tkv* since it failed to complement the loss of function alleles of *tkv* (data not shown). *D2-1* and *D2-3* are potentially new alleles of the *D2* locus, or alternatively, alleles of other lethal loci that may be present on the *D2* chromosome. However, *D2-1* and *D2-3* are likely allelic to *D2* because they are lethal over multiple recombinant chromosomes that still retain the ENU-generated *D2* mutation.

To determine the location of *D2* on the chromosome, its lethality over *D2-1* was meiotically mapped and the results are summarized in Table 4. *D2* mapped to meiotic position between 99 and 100 on the right arm of the second chromosome. Consistent with this mapping data, an inversion breakpoint associated with *D2-3* was found to be in the vicinity of this meiotic position through cytological examination (data not shown).

The enhancer mutations were also tested for their ability to suppress a phenotype generated by expressing a constitutively activated *tkv* receptor ($tkv^{QD}$) (Nellen *et al.*, 1996) in a subset of *vestigial* expression domain using the UAS-Gal4 binary system (Brand and Perrimon, 1993). The ectopic expression of $tkv^{QD}$ in the *vestigial* domain produced wings that were greatly reduced in size and grossly mispatterned with no discernible venation pattern or margin polarity. Heterozygosity of mutations in *Mad* and *D1* partially restored proximal/distal outgrowth as well as the overall pattern of the wings, whereas heterozygosity for mutations in *dpp* or *60A*, which are upstream of *tkv*, failed to suppress the wing phenotype (data not shown). These results are consistent with the role for *Mad* as a

cytoplasmic signal transducer downstream of the *tkv* receptor, and also suggests that like *Mad*, the *D1* gene may encode a component that functions downstream of the *tkv* receptor. A heterozygous *D2* mutation did not give consistent suppression in this assay (data not shown). The preliminary characterization of the *D1* and *D2* enhancer mutations suggests that they potentially define loci that were previously not implicated in *dpp* signaling.

**The *D4* locus corresponds to the *60A* gene, which encodes a BMP-7 homologue**

The *D4* complementation group was represented by three alleles: *D4*, *D8* and *D20*, which all dominantly enhanced the imaginal disc phenotypes of $tkv^6$ homozygotes. Meiotic mapping based on the enhanced phenotypes placed the *D4* complementation group in the distal portion of the right arm of the second chromosome (data not shown). Through further deletion mapping, *D4* was mapped between 60A2 and 60A3 on the polytene chromosome (Figure 4).

To facilitate the molecular cloning of *D4*, attempts were made to recover gamma ray alleles of *D4*. However, nearly 4,000 mutagenized genomes were screened with no recovery of any *D4* alleles. Thus, in order to generate transposon insertional mutations in the *D4* locus, a P element located closely to the interval where D4 was mapped was mobilized. The P element mutagenesis screen was done as outlined in Figure 5. Three independent P element lines that failed to complement *D4* were recovered out of around 4,000 mutagenized genomes. Plasmid rescue experiments were performed to recover the genomic DNA flanking the P element insertion sites (see Materials and Methods). Surprisingly, all P alleles of *D4* as well as the parental P element possessed unique genomic regions on one side of the insertion but shared a common region on the opposite side of the insertion. A genomic map of over 50 kb of the *D4* region was constructed based on the molecular characterization of four overlapping cosmids in the region (Figure 6). When the P element insertion sites were mapped, it was clear that the P alleles of *D4* inserted over a large distance from each other. Based on the observation that they all shared a common flanking genomic sequence on one side, we propose that internal deletion via homologous recombination events occurred between the newly inserted P element and the parental P element. Genomic Southern analysis and the failure to revert the lethality associated with the P insertions by precise excision were consistent with this model.

Southern analysis mapped the *60A* transcript to the small deletion interval predicted to disrupt the *D4* locus, suggesting that *D4* might correspond to the *60A* gene. To confirm that the *D4* locus corresponds to the *60A* gene, the *60A* coding regions from *D4* alleles were sequenced. All three alleles were found to have nonsense mutations in the prodomain of the *60A* polypeptide (Figure 7). Since all three mutations are predicted to eliminate translation of the biologically active mature C-terminal domain, they most likely represent functional nulls of the *60A* gene. In fact, sequencing analysis revealed that mutant $60A^{D4}$ and $60A^{D8}$ mRNAs were greatly under represented in heterozygous animals (Table 5). This could be due to the mRNA instability caused by nonsense mutations (van Hoof and Green,

1996; Kessler and Chasin, 1996; Jenkins *et al.*, 1996). In addition to detecting altered *60A* coding regions in *D4* alleles, we were also able to fully rescue the lethality associated with the *D4* mutations with a *60A* transgene (Table 6). These data prove that the *D4* locus corresponds to the *60A* gene.

**Loss of function phenotypes of *60A***

*60A* gene product is homologous to mammalian proteins BMP-5 and -7, and was identified initially based on sequence homology (Doctor *et al.*, 1992; Wharton *et al.*, 1991). Its function remained largely unknown due to lack of mutations in this gene. The identification of mutations in the *60A* gene allowed analysis of its developmental functions and its role in *dpp* signaling.

Animals lacking *60A* function died at late larval/early pupal stages. One of the striking phenotypes of *60A* mutant larvae is the lack of fat body, which gives them a transparent appearance. In roughly 50% of the *60A* larvae, the gastric caecae were reduced in length, consistent with the expression of *60A* in the gastric caecae (Doctor *et al.*, 1992). These phenotypes are similar to *Mad* mutant larvae (Sekelsky *et al.*, 1995).

During embryogenesis, *60A* is also expressed throughout the visceral mesoderm of the developing midgut (Doctor *et al.*, 1992) suggesting a function for *60A* in the development of the embryonic midgut. Indeed, embryos lacking *60A* failed to form the first constriction. The homeotic gene *Antenapedia (Antp)* is expressed in the visceral mesoderm where the first constriction forms and is required for its formation (Reuter and Scott, 1990; Tremml and Bienz, 1989). We examined *Antp* expression in *60A* mutant embryos. Consistent with the lack of the first constriction, Antp protein was greatly reduced or below detectable level. Thus, *60A* is required for the formation of the first constriction of the midgut, likely through positively regulating the expression of at least one homeotic gene, *Antp* in the visceral mesoderm.

***60A* maintains an optimal level of *dpp* signaling in the visceral mesoderm**

The identification of mutations in *60A* as dominant enhancers of $tkv^6$, thus of *dpp* signaling, in the imaginal discs raised the possibility that *60A* is required for optimal signaling by the *dpp* pathway. To determine if there was a general requirement for *60A* in *dpp* signaling, the effects of *60A* mutations on *dpp* signaling in the visceral mesoderm, where both *dpp* and *60A* are expressed, were examined.

*dpp* is expressed in two distinct domains in the visceral mesoderm (Panganiban *et al.*, 1990b). The anterior expression domain of *dpp* coincides with the gastric caecae primordia, which are immediately anterior to the expression domain of *Sex combs reduced (Scr)* in parasegment (ps) 4. Failure to initiate *dpp* expression in ps3 in $dpp^{shv}$ mutants results in anterior expansion of *Scr* expression and arrest of the outgrowth of the gastric caecae (Panganiban *et al.*, 1990b; Hursh *et al.*, 1993), indicating a role for *dpp* in repressing *Scr* in ps3. $tkv^6$ homozygotes are

homozygous viable, so it is not surprising that all the midgut gene expression patterns examined are essentially normal. *Scr* expression in $tkv^6$ or *60A* mutant animals was normal. However, in embryos that were double mutant for $tkv^6$ and *60A*, the *Scr* expression domain extended anteriorly into ps3, also seen in $dpp^{shv}$ mutants. This suggests that *60A* activity is required in ps3 for optimal *dpp* signaling.

To test whether *60A* acts synergistically with *dpp* elsewhere in the midgut, we examined the gene expression of *dpp* and *Ultrabithorax* (*Ubx*) in ps7 and *wingless* (*wg*) in the adjacent ps8. Expression of *dpp* in ps7 is activated by the homeotic gene *Ubx* (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990b; Reuter *et al.*, 1990; Capovilla *et al.*, 1994; Sun *et al.*, 1995) and maintained by an autostimulatory circuit involving *dpp*, *Ubx* and *wg* (Hursh *et al.*, 1993; Thüringer *et al.*, 1993b; Thüringer *et al.*, 1993a; Staehling-Hampton and Hoffmann, 1994). Proper expression of all three genes is interdependent in order to coordinate a stably maintained cellular differentiation commitment (Bienz, 1994). The expression of *dpp* and *wg* in the visceral mesoderm is required for the induction of the homeotic gene *labial* (*lab*) in the underlying endoderm (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990b; Reuter *et al.*, 1990). The absence of *dpp* function in ps7 disrupts the autoregulatory process and reduces the expression of *Ubx*, *wg* and *dpp* at ps7, leading to the absence of the second constriction in *dpp* mutant embryos (Immerglück *et al.*, 1990; Panganiban *et al.*, 1990b; Reuter *et al.*, 1990).

We found that animals mutant for either $tkv^6$ or *60A* had normal expression of *dpp*, *wg* and *Ubx* . However, in $tkv^6$*60A* double mutants, *dpp* expression in ps3 and ps7 was greatly reduced. The initiation of *dpp* expression at earlier stages was not affected in the double mutants (not shown), suggesting that the reduction of *dpp* expression resulted from failure to maintain its expression at later stages. Similarly, in *Mad* mutants, the initiation of dpp expression in ps3 and ps7 is not affected but the maintenance of *dpp* expression does not occur (Newfeld *et al.*, 1997). This is because *dpp* expression is activated directly by *Ubx* and its maintenance requires positive feedback involving *dpp* signaling. In $tkv^6$*60A* mutants, *Ubx* expression in ps7 and *wg* expression in ps8 were greatly reduced. The reduction of *dpp* in ps3 in the double mutants may explain the derepression of *Scr* observed in these animals.

*Ubx* is required for repressing *Antp* in ps6. In *Ubx* mutants, the *Antp* domain is extended posteriorly into ps7 (Tremml and Bienz, 1989), indicating a homeotic transformation of ps7 into ps6. A similar phenotype was observed for *tkv* null embryos (Affolter *et al.*, 1994). Consistent with the argument that absence of *60A* function compromises *dpp* signaling, there was also a posterior expansion of *Antp* in $tkv^6$*60A* double mutants. Interestingly, the *60A* mutation eliminated the endogenous *Antp* expression and ectopic *Antp* was seen in ps7, where *Ubx* would normally be expressed.

We also examined the expression of *lab*, the target gene of *dpp* signaling in the endoderm. Consistent with the gene expression changes in the visceral mesoderm, *lab* expression was not affected by $tkv^6$ or *60A* mutations. However, it was greatly reduced in $tkv^6$*60A* double mutants. In addition, the gut of the double mutants only formed two-chambers instead of the normal four chambers. This phenotype likely resulted from a failure to form the first constriction due to a lack of *60A* function and a failure to form the second constriction due to a lack of *dpp* signaling. It is unclear why the only constriction observed in the double mutants is positioned somewhat more anterior than a normal third constriction. Taken together with other gene expression changes in the midgut of the $tkv^6$*60A* double mutants, these data provide further evidence for 60A's role in augmenting the signaling capacity of *dpp*.

**_60A_ enhances the ectodermal phenotypes of $tkv^6$ homozygotes**

Previous studies have established *dpp*'s role as a morphogen in patterning the embryonic dorsal epidermis. Higher levels of *dpp* specify more dorsal cell fates, whereas lower levels of *dpp* produce more lateral cell fates (Ferguson and Anderson, 1992a; Wharton *et al.*, 1993). Since the potential for *dpp* to specify cell fates is dosage sensitive, we examined if reducing the dose of *60A* influences cell fate specification mediated by *dpp* signaling.

We compared the cuticle phenotypes of *60A* mutants, $tkv^6$ mutants and $tkv^6$*60A* double mutants. Since $tkv^6$ homozygotes were viable and *60A* mutants had no obvious defects until late in development, the cuticular patterns of these mutants were essentially normal. However, $tkv^6$*60A* homozygotes died as weakly ventralized embryos with head defects and an excessive ventral curvature indicative of defects in germ band retraction and they bear some resemblance to hypomorphic *dpp* mutants(Wharton *et al.*, 1993). This observation suggested that when *dpp* signaling is compromised in the embryonic ectoderm, removing *60A* activity further attenuated *dpp* signaling. The weakly ventralized phenotype of the double mutant is consistent with the loss of the highest level of *dpp* signaling. The relatively mild phenotype may reflect partial rescue by maternally contributed wild type Tkv receptors. Indeed, a quarter of the embryos produced by mothers homozygous for $tkv^6$ and heterozygous for *60A* exhibited a dorsal open phenotype similar to that of the zygotic *tkv* null embryos (Penton *et al.*, 1994).

**Table 1. Summary of enhancer mutations**

| Gene | enhanced phenotype | chromosomal location | number of alleles | allelic to |
|---|---|---|---|---|
| *D1* | notum | second | 1 | - |
| *D2* | notum | second | 1 | - |
| *D3* | legs and notum | second | 5 (*D3, D14, D15, D16, D24*) | *Mad* |
| *D17* | legs and notum | second | 1 | *tkv* |
| *D4* | legs and notum | second | 3 (*D4, D8, D20*) | *60A* |
| *D5* | legs and notum | third | 1 | *Med* |
| *D13* | legs and notum | third | 2 (*D13, D18*) | *punt* |

The assignment of the enhancer mutations to different genes (complementation groups) is based on genetic mapping and *inter se* complementation tests among enhancer mutations as described in Experimental Methods.

**Table 2 Heterozygous interactions between enhancer mutations and *dpp* pathway mutations**

| | $tkv^6$ | *Df(2L) tkv2* | $dpp^{s5}$ | $shn^{IB}$ | $sax^5$ | $Mad^P$ | $Med^4$ | *punt 135* |
|---|---|---|---|---|---|---|---|---|
| $tkv^6$ | -# | -# | - | - | - | - | - | - |
| $tkv^6D1$ | 67 | 71 | 64 | 60 | - | - | 31 | 40 |
| $tkv^6D2$ | 43 | 53 | 34 | - | 31 | - | - | 41 |
| $tkv^6D3$ | 94* | 100* | 96 | 100* | - | lethal | 23 | 68 |
| $tkv^6D15$ | 96* | 91* | 100 | 100* | - | lethal | 29 | 78 |
| $tkv^6D24$ | 100* | 100* | 100 | 100* | - | lethal | 41 | 81 |
| $tkv^6D14$ | 98 | lethal | 68 | - | 44 | lethal | - | 35 |
| $tkv^6D16$ | 31 | 50 | - | - | 41 | 48 | - | - |
| $tkv^6D17$ | 65 | lethal | - | 15 | 68 | - | - | - |
| $tkv^6D4$ | 78 | 83 | 37 | 43 | - | - | - | 25 |
| $tkv^6D8$ | 82 | 87 | 31 | 39 | - | - | - | 22 |
| $tkv^6D20$ | 68 | 73 | 25 | 50 | - | - | - | 31 |
| *D5* | 47 | 52 | ND | ND | ND | ND | lethal | - |
| *D13* | 61 | 63 | ND | ND | ND | ND | - | lethal |
| *D18* | 62 | 67 | ND | ND | ND | ND | - | lethal |

Numbers represent the percentage of heterozygous progeny with disk phenotypes such as gaps in wing veins, cleft in the notum and/or distal truncation of the legs (lethal, failure to recover the indicated progeny class; asterisk, severe reduction of the indicated progeny class to less than 10% of the expected number; minus, no interactions observed; ND, not done; #, thickened wing veins only, due to non-complementation with *tkv* alleles).

**Table 3. Maternal effect of the enhancer mutations on the zygotic viability of *dpp$^{hr27}$***

| Maternal genotype | Wild type wings | Held out wings | Percent of expected |
|---|---|---|---|
| *tkv$^{7}$/dpp$^{hr27}$* | 283 | 3 | 3% |
| *tkv$^{6}$/dpp$^{hr27}$* | 177 | 32 | 54% |
| *tkv$^{6}$D1/dpp$^{hr27}$* | 112 | 3 | 8% |
| *tkv$^{6}$D2/dpp$^{hr27}$* | 200 | 13 | 20% |
| *tkv$^{6}$D4/dpp$^{hr27}$* | 210 | 38 | 54.3% |
| *tkv$^{6}$D20/dpp$^{hr27}$* | 238 | 40 | 50.4% |
| *tkv$^{6}$D14/dpp$^{hr27}$* | 179 | 0 | 0% |
| *tkv$^{6}$D16/dpp$^{hr27}$* | 87 | 3 | 10% |
| *tkv$^{6}$D17/dpp$^{hr27}$* | 208 | 17 | 24.5% |

Females of the genotypes in column one were generated by mating heterozygous *dpp$^{hr27}$* females to males heterozygous for the indicated mutations. Females with the indicated maternal genotypes were then mated to males that had a *dpp$^{d\text{-}ho}$* mutant chromosome (*dpp$^{H32}$ Sp Bl Dp(2;2)dTD48 dpp$^{d\text{-}ho}$/In(2LR)Gla Bc*). Of the four progeny classes, three had wild type wings. The *dpp$^{hr27}$* and the *dpp$^{d\text{-}ho}$* mutations failed to complement and produced heldout wings. Thus the heldout phenotype was used as an indicator for the zygotic viability of *dpp$^{hr27}$*.

**Table 4 Meiotic mapping of *D2* based on its lethality in trans to *D2-1***

| Recombinant classes | Numbers of progeny in the class |
|---|---|
| *pr cn D2* | 229 |
| *pr cn* + | 178 |
| + + *D2* | 175 |
| + + + | 238 |
| *pr* + *D2* | 4 |
| *pr* + + | 10 |
| + *cn D2* | 14 |
| + *cn* + | 7 |
| Total number of recombinants | 855 |

The genetic crosses used to generated these data are described in the Materials and Methods.

Recombinant frequencies (RF) between loci:

*pr cn* RF = (4+10+14+7)/855 = 4 (The published distance is 3)

*cn D2* RF = (178+7+175+4)/855 = 42

*pr D2* RF = (178+10+175+14+7x2+4x2)/855 = 46

Based on the published meiotic map locations of *pr* (54.5) and *cn*(57.5), the *D2* locus was calculated to be between 99 (42+57.5) and 100 (46+54.5). This calculation does not take into consideration the difference in the published and observed value for the distance between *pr* and *cn*. Since the distance between the putative *D2* locus and *pr* or *cn* is very large, the RF can substantially deviate from the actual map distance due to multiple crossing over events. Thus the mapping data presented here should be used with caution.

**Table 5 Mutation spectrum of the ENU alleles sequenced**

| Mutant alleles | Mutant clones/ Total clones sequenced | Codon changes | Nature of the mutations |
|---|---|---|---|
| *Mad*$^{D3}$ | 7/15 | No mutations in the coding region | |
| *Mad*$^{D15}$ | 5/14 | No mutations in the coding region | |
| *Mad*$^{D24}$ | 7/11 | GAC to AAC | G:C to A:T transition |
| *Mad*$^{D14}$ | 5/11 | CAG to TAG | G:C to A:T transition |
| *Mad*$^{D16}$ | 3/7 | TAT to AAT | A:T to T:A transversion |
| *punt*$^{D13}$ | 18/28 | TGC to TAC | G:C to A:T transition |
| *punt*$^{D18}$ | 7/13 | GAG to GTG | A:T to T:A transversion |
| *60A*$^{D4}$ | 1/14 (cDNA)<br>4/10 (genomic) | One base (C) deletion causes a frame-shift nonsense mutation | |
| *60A*$^{D8}$ | 0/12 (cDNA)<br>7/14 (genomic) | TGG to TGA | G:C to A:T transition |
| *60A*$^{D20}$ | 5/17 | CAG to TAG | G:C to A:T transition |

See Materials and Methods for the sequence analysis procedures. The mutant clones were identified by comparing chromosome-specific polymorphisms from multiple alleles generated in the same genetic background.

**Table 6. Rescue of the lethality of the *D4* complementation group by a *60A* transgene**

| Number of progeny of the designated genotypes | | |
|---|---|---|
| | *p[w+ 6.5sal]1.2/+* | +/+ |
| *D8/tkv$^6$ pr cn D4 (Cy+)*[a] | 32 (84%) | 0 |
| *D8* or *tkv$^6$ pr cn D4/CyO* [a] | 76 | 78 |
| *D20/tkv$^6$ pr cn D4 (Cy+)*[b] | 69 (>100%) | 0 |
| *D20 or tkv$^6$ pr cn D4/CyO* [b] | 83 | 91 |
| *Df((2R)bw$^{Dra}$/tkv$^6$ pr cn D4 (Cy+)*[c] | 37 (98%) | 0 |
| *Df(2R)bw$^{Dra}$ or tkv$^6$ pr cn D4/CyO*[c] | 75 | 90 |

*p[w+ 6.5sal]1.2* is a transgene carrying 6.5 kb of genomic DNA containing *60A* as well as two other coding sequences, *peter pan* and *sahara* (Wharton, K. personal communication). *D4, D8* and *D20* fully complemented a small deficiency (*Dr(2R)P10-107*) that deletes *peter pan* and *sahara* (data not shown), indicating that they are not allelic to either *peter pan* or *sahara*.

The progeny derive from the following crosses:

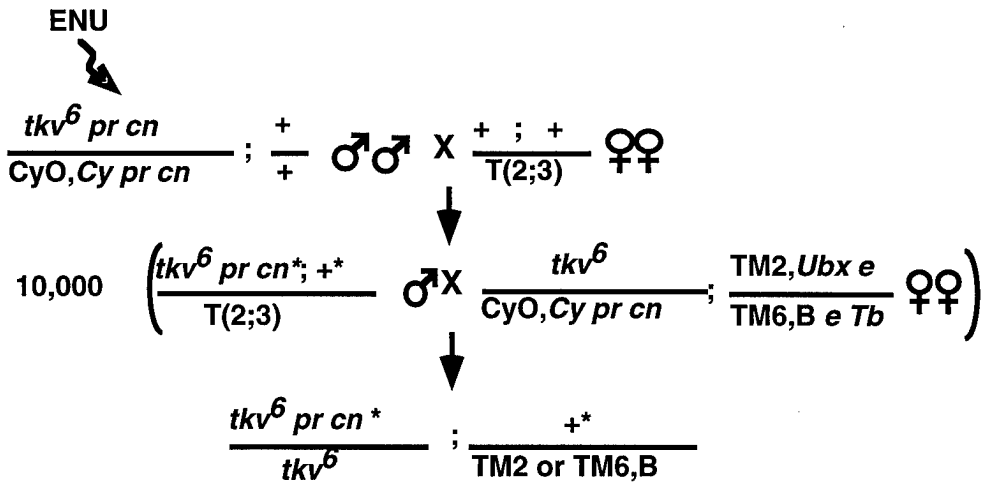
[a] *tkv$^6$ pr cn D4/CyO; p[w+ 6.5sal]1.2/+* X *D8/CyO; +/+*

[b] *tkv$^6$ pr cn D4/CyO; p[w+ 6.5sal]1.2/+* X *D20/CyO; +/+*

[c] *tkv$^6$ pr cn D4/CyO; p[w+ 6.5sal]1.2/+* X *Df(2R)bw$^{Dra}$/CyO; +/+*

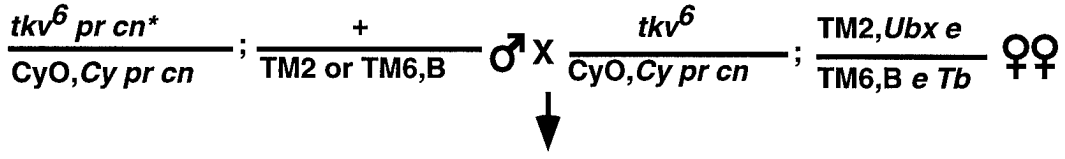
The first column and row describe the genotypes of the second chromosome and third chromosome, respectively. The number in parentheses are the percentages of expected *Cy+* progeny rescued to adulthood by one copy of the transgene.

**Figure 1. Scheme for the F2 enhancer screen of *tkv*$^{6}$.**
ENU, Ethylnitrosourea; *T(2;3)*; a translocation between *CyO* chromosome and *TM6,B* chromosome which forces the cosegregation of the second and third chromosome. Asterisks indicate mutagenized chromosomes. See Experimental Methods for detailed description of full genotypes and the procedure.



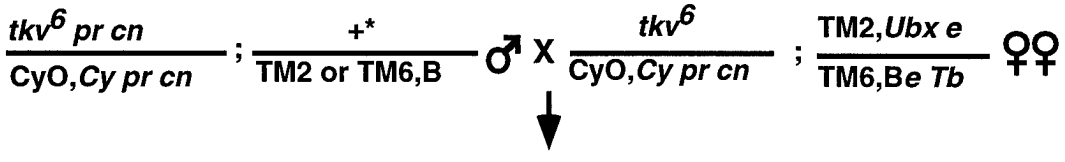

score for disk phenotype modifiers and map chromosomal location

**1. For modifiers on the second chromosome**




retest for modification and make balanced stock

**2. For modifiers on the third chromosome**




retest for modification and make balanced stock

**Figure 2. Molecular lesions in the new alleles of *Mad* and *punt*.**
The position and the amino acid changes of each mutation are indicated below the schematic diagram of the protein. Asterisks represent nonsense mutations. (A) Mutations in new *Mad* alleles. Closed bars represent the highly conserved Mad Homology domain 1 and 2 (MH1 and MH2). The hatched box at the Carboxy terminal represents the mutation hot spot. (B) Mutations in new *punt* alleles. The structural features of the punt Type II receptor shown are the putative signal peptide (oval box), the extracellular cysteine residues (vertical bars), the transmembrane domain (hatched box) and the kinase domain (open box).

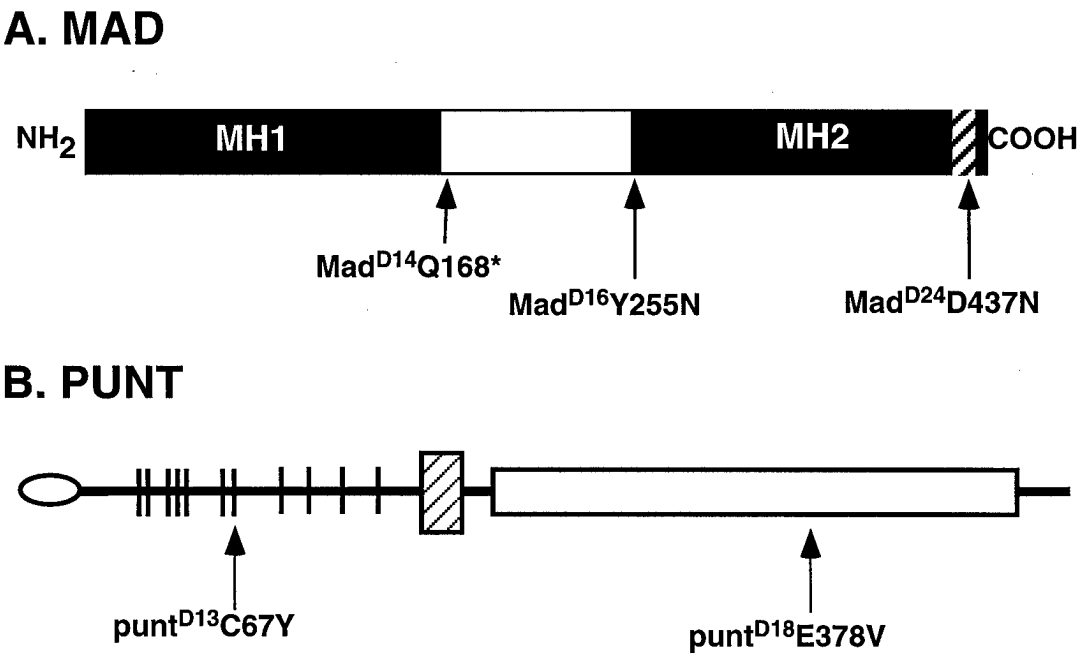


**Figure 3. Scheme for the gamma ray screen for *D2* alleles.**
The asterisk labels the chromosomes exposed to the gamma ray irradiation. See Experimental Methods for a more detailed description of the procedure.

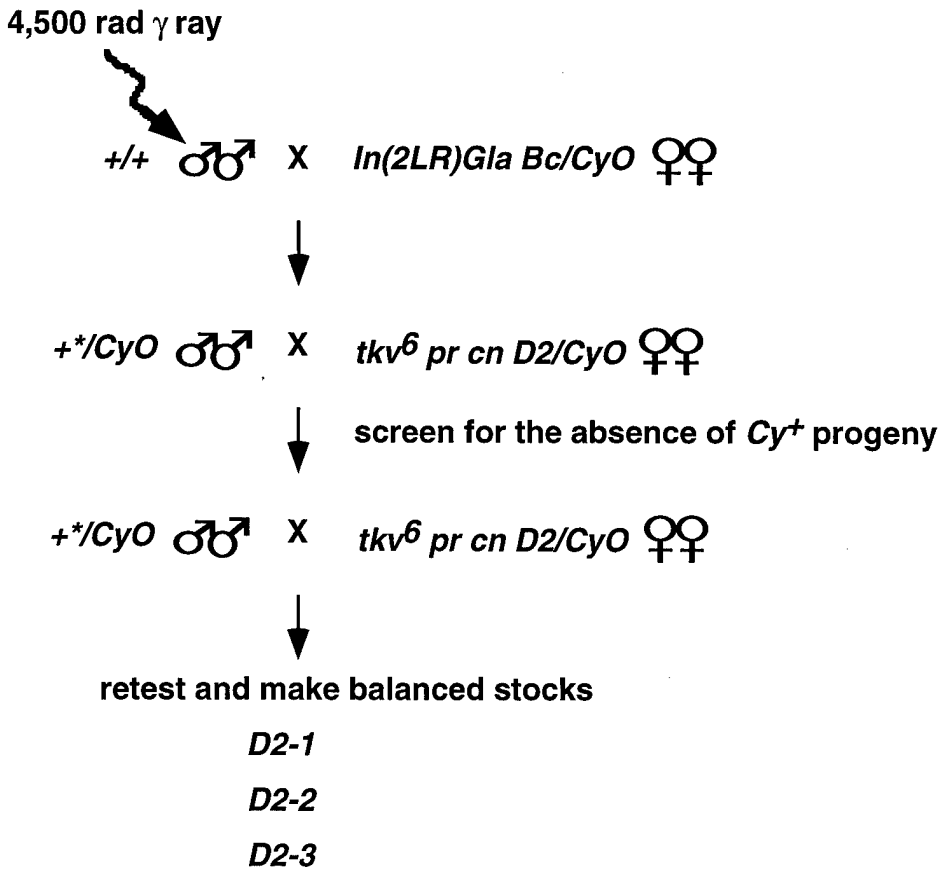


**Figure 4 Deletion mapping of the *D4* locus.**
Shown is the polytene chromosome region of 60A. Numbers above the polytene chromosome represent the subdivisions within 60A. Deficiency stocks used for the mapping are listed below the polytene map with the names and the regions deleted indicated. Open or hatched boxes represent the complementing or non-complementing deficiencies respectively. The region between the vertical dashed lines represent the interval to which D4 was mapped.

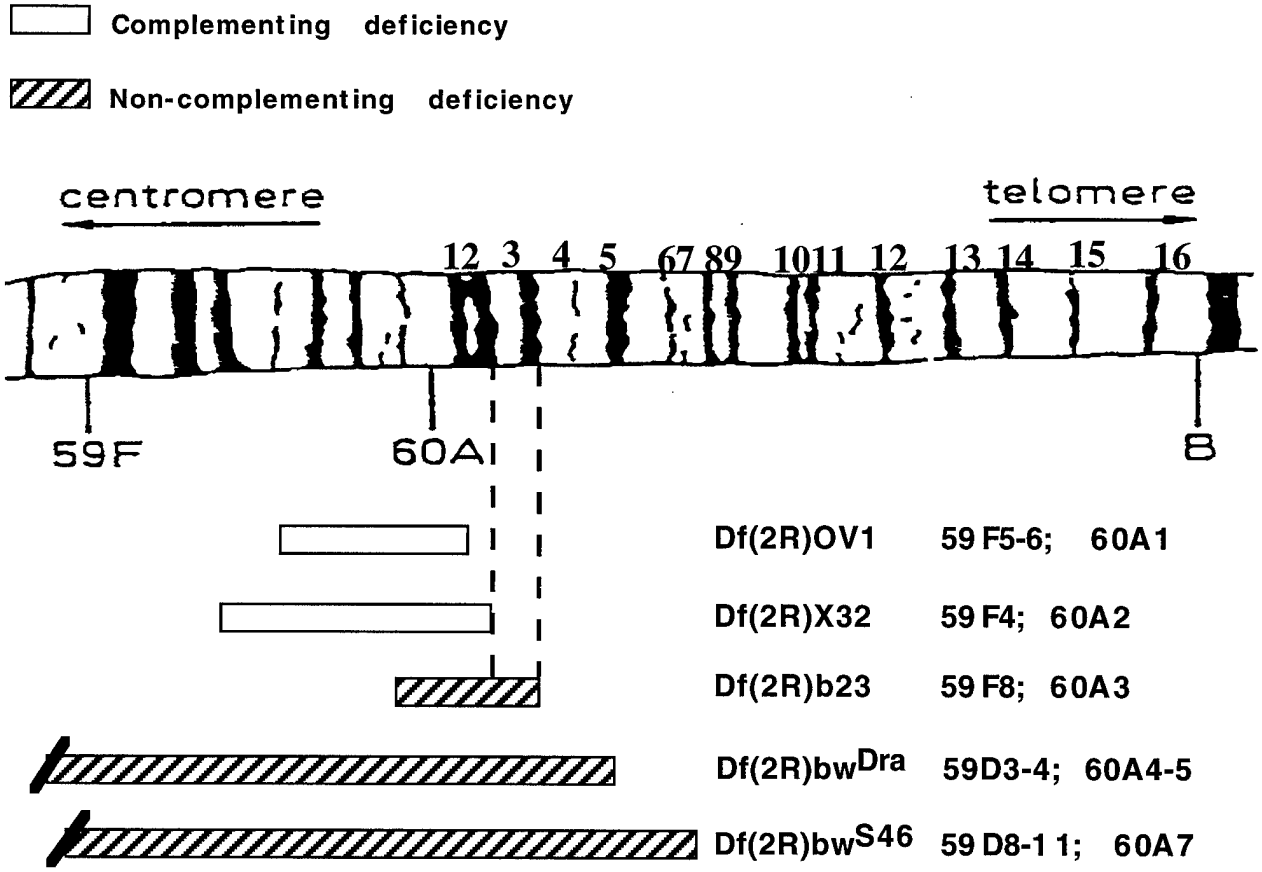


**Figure 5. Scheme for the P element mutagenesis of *D4*.**
Δ2-3 represents the source of the transposase used in the screen. The details of the procedure of the mutagenesis are described in Experimental Methods.

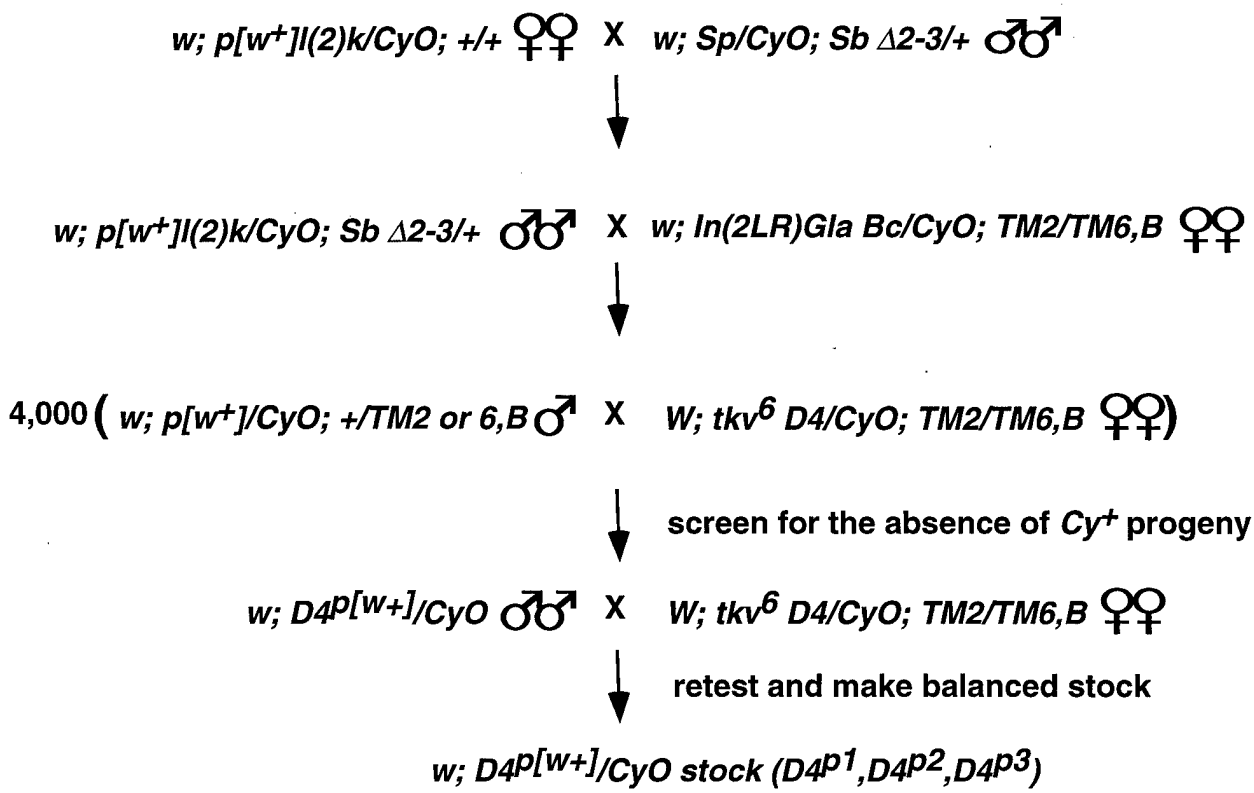


**Figure 6. The genomic organization of the *D4* locus.**
The genomic map of the *D4* region is shown with XbaI (X) and NotI (N) restriction enzyme sites indicated. The closed triangle represents the parental P element, l(2)k, of which the new P alleles of *D4* (open triangles) are derived. The hatched boxes represent the genomic fragment rescued from the left side of the insertion using the EcoRI restriction enzyme. The closed box represents the genomic fragment rescued from the right side of the insertion using the BglII restriction enzyme. Southern and sequence analysis indicated that the BglII rescued fragment from all the P alleles of *D4* were identical to that of the parental P element. The overlapping cosmids isolated using the EcoRI rescued fragment of *D4-P2* are shown below the genomic map. The *60A* transcript is indicated with a bracket.

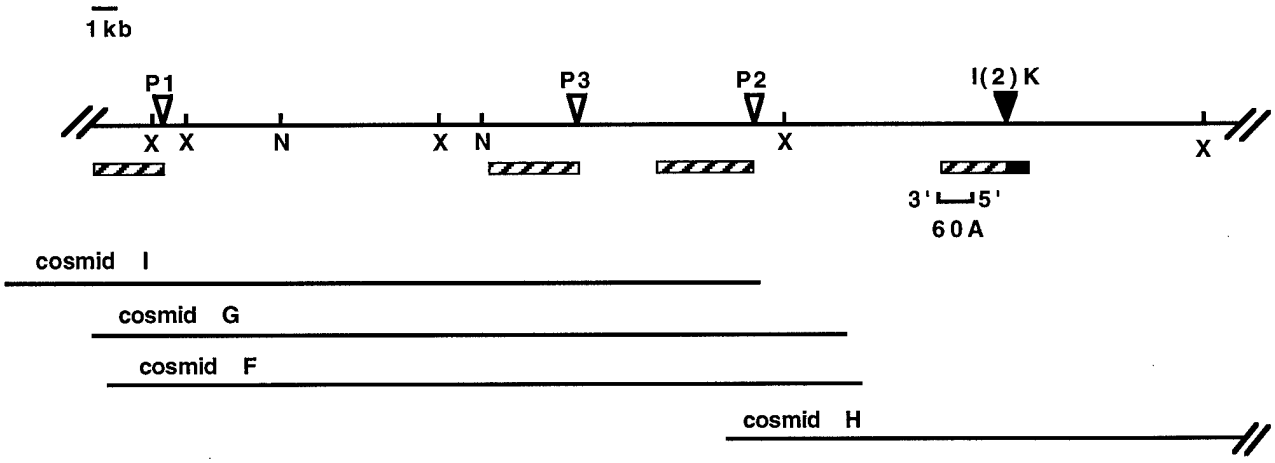
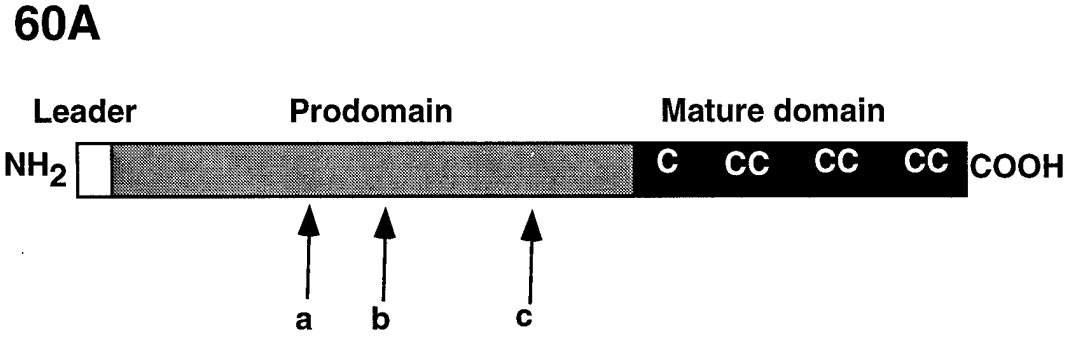


**Figure 7. Mutations in *D4* alter the *60A* coding region.**
Shown is a schematic representation of the 60A polypeptide with the structural features indicated. The seven Cs within the mature domain represent the seven conserved cysteines. The arrows point to the position of the mutations in the coding region. The molecular lesions are indicated. $60A^{D8}$ and $60A^{D20}$ are nonsense mutations resulting from single nucleotide substitutions. $60A^{D4}$ is a frame-shift nonsense mutation resulting from a single nucleotide deletion.




a. $60A^{D20}$ Q137*

b. $60A^{D8}$ W179*

c. $60A^{D4}$ V237* (frame shift nonsense mutation)

## DISCUSSION

The haploinsufficiency of the *dpp* locus reflects the sensitivity of developmental processes to a reduction in *dpp* signaling. We have carried out a genetic screen to search for modifiers of $tkv^6$, a hypomorphic allele of the *tkv* gene, which encodes a type I receptor for *dpp*. *tkv* has been implicated in all aspects of *dpp* signaling both *in vitro* and *in vivo* (Penton *et al.*, 1994; Nellen *et al.*, 1994; Brummel *et al.*, 1994; Affolter *et al.*, 1994; Burke and Basler, 1996a; de Celis, 1997; Singer *et al.*, 1997). Thus the modifiers of $tkv^6$ are likely to be integral components of the *dpp* signal transduction pathway. In fact, new alleles of several genes known to mediate *dpp* signaling were recovered. These included *tkv* , *punt*, *Mad* and *Med*. These mutations dominantly enhanced the mild imaginal disc phenotypes of $tkv^6$. Recovery of these mutations validated the specificity of the screen.

Five new alleles of Mad, a key signal transducer in *dpp* signaling (Newfeld *et al.*, 1996; Kim *et al.*, 1997) were isolated. Three have point mutations in the coding region (Figure 3.4A). The molecular lesions correlate with their phenotypic properties. $Mad^{D14}$ has a nonsense mutation predicted to produce a truncated protein with only the conserved MH1 domain and it behaves genetically as a null. $Mad^{D16}$ has a tyrosine to asparagine change in the divergent linker region and behaves genetically as a hypomorph, suggesting that the amino acid change only partially affects the protein function. The MH2 domains of Mad-related proteins (Smads) are highly conserved. The three dimensional structure of the MH2 domain of Smad4 indicates that it mediates the homotrimerization of Smad proteins. The intact conformation of the homotrimer is essential for the assembly of a heterohexamer with another Smad homotrimer in response to receptor activation (Hata *et al.*, 1997; Shi *et al.*, 1997). Many of the Smad mutations associated with tumors or developmental defects map within the MH2 domain. Based on the crystal structure of the carboxy terminal domain of Smad4 (Shi *et al.*, 1997), the invariant aspartic acid mutated to asparagine in $Mad^{D24}$ maps to the trimer interface region, which is critical for trimerization. The corresponding residue in Smad2 is mutated in colon cancers (Eppert *et al.*, 1996). Interestingly, the $Mad^{D24}$ mutation resulted in dominant female sterility, which is not observed with *Mad* null alleles. This suggests that the $Mad^{D24}$ mutation has a dominant negative effect, perhaps by forming inactive oligomers with the wild type protein in the heterozygotes.

Analysis of the two new *punt* alleles also provides evidence for the *in vivo* importance of conserved structural motifs in this type II *dpp* receptor. $punt^{D13}$ has a cysteine to a tyrosine change in the extracellular cysteine cluster characteristic of all receptors for TGF-β superfamily members (Massagué *et al.*, 1994). $punt^{D18}$ changes the highly conserved glutamic acid to a valine in the catalytic core of the kinase domain (Hanks *et al.*, 1988), where another punt mutation, $punt^{135}$ has been

mapped (Ruberte *et al.*, 1995). Like *punt*$^{135}$, both new *punt* alleles display some temperature sensitivity, suggesting that they are not protein nulls. To date, no null mutations in the *punt* locus have been reported, suggesting that like *dpp*, *punt* may be haploinsufficient.

Besides providing information about the structure-function relationship, the sequencing data also added useful information on the germline mutation spectrum of ENU. Of eight ENU alleles sequenced that had mutations in the respective coding regions, five were GC to AT transitions, two were AT to TA transversions and one involved a single nucleotide deletion. The frequencies of the two types of nucleotide substitution is comparable to the reported frequencies of the ENU generated mutations in *Drosophila* (see references in Marker *et al.*, 1997). Although ENU generated deletions were not previously reported in *Drosophila*, they were recovered at a low frequency in *E. coli* and mice (see references in Marker *et al.*, 1997).

No new alleles of *shn* were isolated although it acted as an enhancer of *tkv*$^{6}$ in the initial test. The enhancement by *shn*$^{IB}$ may be allele specific, such that a particular form of Shn mutant protein is needed to produce an enhancement effect. Consistent with this, *shn*$^{p}$, which makes no detectable protein (Staehling-Hampton *et al.*, 1995), failed to enhance the *tkv*$^{6}$ phenotype (data not shown). No *dpp* alleles were recovered either, possibly due to the haploinsufficiency associated with the locus and the fact that most hypomorphic *dpp* mutations affect the regulatory regions of *dpp*, which are less likely to be affected by chemical mutagens such as ENU.

Preliminary characterization of *D1* and *D2* indicates that they potentially define two new loci. Both mutations have extensive genetic interactions with other components of the *dpp* pathway. Heterozygous interactions between recessive mutations are rare and are usually observed for genes that encode proteins involved in the same pathway and sometimes may reflect a physical association between the proteins. Thus the heterozygous interactions between these two enhancers and the *dpp* pathway mutations argues for their role(s) in the *dpp* pathway. In addition, the *D1* mutation maternally enhanced the zygotic lethality of *dpp*$^{27}$. The suppression of a phenotype associated with an activated *tkv*$^{QD}$ by heterozygous *D1* mutation suggests that it may act downstream of *tkv*. However, the epistatic relationships inferred from this experiment should be taken with caution because it not known whether the *tkv*$^{QD}$ receptor is fully activated or not. If it is not fully activated, then it would be possible to achieve suppression by a quantitative reduction in the activity of an upstream component. More thorough characterizations of these two loci are in progress to determine their molecular identity and role(s) in the *dpp* pathway.

Mutations in the *60A* gene, which encodes the *Drosophila* homologue of mammalian BMP-5 and -7 (Doctor *et al.*, 1992; Wharton *et al.*, 1991) were identified in a modifier screen for components involved in *dpp* pathway. The fact that in a screen of the entire genome, *60A* mutations were recovered multiple times as dominant enhancers of a mutant *dpp* receptor provides strong evidence for its involvement in *dpp* signaling.

Phenotypic analysis of *60A* single mutants and $tkv^{6}$*60A* double mutants revealed both *dpp*-independent and *dpp*-dependent functions for *60A*. 60A protein is expressed in a broad pattern throughout development, but is enriched in the developing gut region (Doctor *et al.*, 1992), suggesting a role for *60A* in gut morphogenesis. *60A* mutants lack the first constriction of the embryonic midgut and *Antp* expression in ps6. This suggests that *60A* is required for the formation of the first constriction, possibly through regulating *Antp* expression. This function is independent of *dpp* signaling, since mutations in *dpp* and its receptors only disrupt the formation of the second constriction. Neither *tkv*, *sax* nor *punt* affects the formation of the first constriction, suggesting that there is either redundancy or that a different receptor system may be responsible for mediating *60A* signaling to pattern the first constriction. It would be interesting to see if AtrI (Childs *et al.*, 1993) , a type I receptor and STK-D (Ruberte *et al.*, 1995), a type II receptor in *Drosophila*, both of unknown function, are mediators of *60A* signaling at the site of the first constriction.

The role of *60A* in potentiating dpp signaling in revealed by the fact that *60A* mutations are dominant enhancers of a sensitized *dpp* pathway. This is most obvious in the visceral mesoderm of the midgut where *dpp* signaling is required to regulate homeotic gene expression and to positively stimulate its own expression through an autocrine mechanism. Although *dpp* signaling in the visceral mesoderm appears intact in *60A* mutants, a requirement for *60A* is revealed in the $tkv^{6}$*60A* double mutants. These double mutants show derepression of *Scr* in the anterior midgut and the loss of expression of the *dpp* target genes, *wg*, *Ubx* and *dpp*, in the visceral mesoderm and *lab* in the underlying endoderm, consistent with the loss of positive feedback on *dpp*. A similar requirement for *60A* is observed in the dorsal/ventral patterning of the embryonic ectoderm. The enhanced phenotypes of the adult appendages closely resemble those of *dpp* hypomorphic mutations (Spencer *et al.*, 1982), suggesting that *60A* activity is also required in imaginal disc patterning. It is interesting that the imaginal discs are more sensitive to reduction of *60A* function, as a 50% reduction in *60A* function is sufficient to produce a phenotype in a $tkv^{6}$ genetic background. This may reflect a differential threshold requirement for *dpp* signaling in different tissues. Taken together, our data argue for an involvement of *60A* in *dpp* signaling at different developmental stages and in various tissues.

In a signaling system with multiple interacting ligands, the interpretation of any single mutant phenotypes must consider the effect of losing both homomeric

and possible heteromeric ligands. Therefore, the functions of the *dpp* pathway may well be a composite input from Dpp homodimers, Dpp/Scw and Dpp/60A heterodimers. An alternative model is that 60A homodimers function in an additive fashion with Dpp homodimers at sites of overlapping expression. However, the loss of function phenotypes of *dpp* are as severe as the loss of function phenotypes of its downstream components, such as *tkv* or *Mad* (Padgett *et al.*, 1987; Penton *et al.*, 1994; Nellen *et al.*, 1994; Newfeld *et al.*, 1996; Newfeld *et al.*, 1997), suggesting that there is very little requirement for *60A* signaling, if any at all, from 60A homodimers in *dpp*-dependent events. Therefore, we believe it is unlikely that 60A homodimers play a significant role in *dpp* dependent processes. Rather, we favor the interpretation that Dpp/60A heterodimers form at sites of overlapping expression and participate with Dpp homodimers in multiple signaling events.

The overlapping expression patterns of multiple murine BMPs have led to the suggestion that they may act combinatorially during development (Lyons *et al.*, 1995). Given the dimeric nature of the TGF-β superfamily ligands, one mechanism to achieve such a combinatorial effect is to form functional heterodimers. Heterodimers of *Xenopus* BMP-4 and -7 have been generated *in vitro* (Hazama *et al.*, 1995) and have been shown to be more potent in bone (Aono *et al.*, 1995) and mesoderm inducing assays (Suzuki *et al.*, 1997) than either homodimer. In *Drosophila*, another BMP-related factor, Scw, is proposed to up-regulate the activity of Dpp by forming Dpp/Scw heterodimers in the dorsal/ventral patterning of the embryonic ectoderm (Arora *et al.*, 1994). The broad distribution of 60A proteins provides an opportunity for forming Dpp/60A heterodimers. Unlike *scw* null mutations, no obvious disruption of *dpp* signaling is observed in *60A* null mutants, suggesting that Dpp/60A heterodimers are not as limiting as Dpp/Scw heterodimers, but partially redundant with Dpp homodimers. The first constriction phenotype of 60A mutants is unique, suggesting that it may be a function of 60A homodimers.

The $tkv^6$ receptor failed to bind homomeric ligands when expressed in COS cells (Penton *et al.*, 1994), although *dpp* signaling is largely intact in $tkv^6$ mutants. Thus, it would be interesting to determine if the mutant receptor still binds Dpp/60A and/or Dpp/scw heterodimers. If the $tkv^6$ mutation specifically reduces homodimer binding but had little or no effect on heterodimer binding, it would indicate that Dpp/60A heterodimers are capable of providing sufficient signaling in the absence of signaling from Dpp homodimers. The severe midgut phenotypes of $tkv^6$*60A* double mutants could be due to additional loss of signaling from Dpp/60A heterodimers. This would be consistent with our hypothesis that *60A* potentiates *dpp* signaling by forming heterodimers with Dpp. The relatively mild cuticular defects of $tkv^6$*60A* double mutants lacking maternally provided wild type Tkv receptors may be due to residual Dpp/Scw heterodimer signaling through the mutant $tkv^6$ receptor during early embryogenesis.

## CONCLUSIONS

The genetic approach supported by the grant worked. New mutations were recovered in virtually every known component of the dpp signaling pathway. The importance of another ligand, the BMP7 homolog 60A, in dpp signaling was discovered for the first time. A primary goal was to discover new components and to use the Drosophila genes as probes to identify the human genes and study their role, if any, in human breast cancer. *D1* and *D2* are $tkv^6$ enhancer mutations that potentially represent two novel loci. They interact extensively with other mutations in the *dpp* pathway. *D1*, in particular, is able to partially suppress a dominant phenotype generated by expressing a constitutively active *tkv* receptor in the wing, suggesting that it may act downstream of the receptor. On-going experiments indicate that *D1* may be a mutation in dpp itself or in a very closely linked gene. The focus of on-going efforts has shifted to *D2*. We want to know what kind of protein *D2* encodes and to understand its role in *dpp* signal transduction. The location of *D2* has been determined by meiotic and deletion mapping. Pre-existing mutations have been tested for possible allelism but no other alleles were found. One other allele was obtained from the laboratory of Dr. Richard Padgett, Rutgers University. It was recovered there independently by a different modifier strategy. DNA from the region was obtained as a cosmid contig from the European Drosophila Genome Project. Current expermeints are seeking to identify which transcript is altered by the *D2* mutations.

It is likely that there are additional factors important for the regulation of the *dpp* pathway that were not uncovered by the modifier screen accomplished in this grant period. For example, there may be a cytoplasmic sequestering factor regulating the nuclear translocation of Mad proteins and potential transcriptional partner(s) of Mad, like FAST-1 in *Xenopus* (Chen *et al.*, 1996). In addition to the general signal transducers, there may be tissue specific effectors of the *dpp* pathway. Since the $tkv^6$ background has been proven to be a low-noise background and only 10,000 mutagenized genomes have been screened, it is possible that scaling up the screen, would unveil more modifier loci. Also, the dominant female sterility associated with some *Mad* alleles recovered in the screen reported here may be a useful genetic background to screen for mutations that would revert the dominant female sterility. Finally, one can look for suppressors of the wing phenotypes caused by expressing the constitutively active *tkv* in the *vestigial* pattern. Possible suppressors of this phenotype may be loss of function mutations in the positive regulators of the *dpp* pathway or activating mutations in the factors that negatively regulate the pathway.

The remarkably diverse biological activities elicited by TGF-β-related factors confers clinical potential for treating cancer, stimulating tissue repair and modulating development. It is very satisfying to realize that what we have learned in this tiny red-eyed creature has had and will continue to have a profound impact on our understanding of similar biological processes present in humans.

## REFERENCES


Affolter, M., Nellen, D., Nussbaumer, U., and Basler, K. (1994). Multiple requirements for the receptor serine/threonine kinase *thick veins* reveal novel functions of TGF-β homologs during *Drosophila* embryogenesis. Development *120*, 3105-3117.

Alexandrow, M. G. and Moses, H. L. (1995). Transforming growth factor β and cell cycle regulation. Cancer Research *55*, 1452-1457.

Aono, A., Hazama, M., Notoya, K., Taketomi, S., Yamasaki, H., Tsukuda, R., Sasaki, S., and Fujisawa, Y. (1995). Potent ectopic bone-inducing activity of bone morphogenetic protein-4/7 heterodimer. Biochem. Biophys. Res. Commun. *210*, 670-677.

Arora, K., Dai, H., Kazuko, S. G., Jamal, J., O'Connor, M. B., Letsou, A., and Warrior, R. (1995). The *Drosophila schnurri* gene acts in the Dpp/TGF-β signaling pathway and encodes a transcription factor homologous to the human MBP family. Cell *81*, 781-790.

Arora, K., Levine, M. S., and O'Connor, M. B. (1994). The *screw* gene encodes a ubiquitously expressed member of the TGF-β family required for specification of dorsal cell fates in the *Drosophila* embryo. Genes Dev. *8*, 2588-2601.

Ashburner, M. (1989). *Drosophila:* A Laboratory Manual, (New York: Cold Spring Harbor Laboratory Press).

Attisano, L. and Wrana, J. L. (1996). Signal transduction by members of the transforming growth factor-β superfamily. Cytokine Growth Factor Rev. *7*, 327-339.

Attisano, L., Wrana, J. L., Lopez-Casillas, F., and Massagué, J. (1994). TGF-β receptors and actions. Biochim. Biophys. Acta. *1222*, 71-80.

Baker, J. C. and Harland, R. M. (1996). A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. Genes Dev. *10*, 1880-1889.

Baker, N. E. (1988). Transcription of the segment-polarity gene *wingless* in the imaginal discs of *Drosophila* and the phenotype of a pupal lethal *wg* mutation. Development *102*, 489-497.

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of *Drosophila* limb pattern by *hedgehog* protein. Nature *368*, 208-214.

Bienz, M. (1994). Homeotic genes and positional signalling in the *Drosophila* viscera. Trends Genet. *10*, 22-26.

Bienz, M. (1996). Induction of the endoderm in *Drosophila*. Semin. Cell Dev. Biol. *7*, 113-119.

Bier, E., Vaessin, H., Shepherd, S., Lee, K., McCall, K., Barbel, S., Ackerman, L., Carretto, R., Uemura, T., Grell, E., Jan, L. Y., and N., J. Y. (1989). Searching for pattern and mutation in the *Drosophila* genome with a P-*lac*Z vector. Genes Dev. *3*, 1273-1287.

Blackman, R. K., Sanicola, M., Raftery, L. A., Gillevet, T., and Gelbart, W. M. (1991). An extensive 3' cis-regulatory region directs the imaginal disk expression of *decapentaplegic*, a member of the TGF-β family in *Drosophila*. Development *111*, 657-665.

Blair, S. (1995). Compartments and appendage development in *Drosophila*. BioEssays *17*, 299-309.

Boyd, F. T. and Massagué, J. (1989). Growth inhibitory response to transforming growth factor-β linked to expression of a 53 KDa cell surface TGF-β receptor. J. Biol. Chem. *264*, 2272-2278.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Brook, W. J. and Cohen, S. M. (1996). Antagonistic interactions between *wingless* and *decapentaplegic* responsible for dorsal-ventral pattern in the *Drosophila* leg. Science *273*, 1373-1377.

Brook, W. J., Diaz-Benjumea, F. J., and Cohen, M. S. (1996). Organizing spatial pattern in limb development. Annu. Rev. Cell Dev. Biol. *12*, 161-180.

Brummel, T. J., Twombly, V., Marqué, G., Wrana, J. L., Newfeld, S. J., Attisano, L., Massagué, J., O'Connor, M. B., and Gelbart, W. M. (1994). Characterization and relationship of Dpp receptors encoded by the *saxophone* and *thick veins* genes in *Drosophila*. Cell *78*, 251-261.

Bryant, P. J. (1975). Pattern formation in the imaginal wing disc of *Drosophila melanogaster*: fate map, regeneration, and duplication. J. Exp. Zool. *193*, 49-78.

Bryant, P. J. (1988). Localized cell death caused by mutations in a *Drosophila* gene coding for a transforming growth factor-β homolog. Dev. Biol. *128*, 386-395.

Burke, R. and Basler, K. (1996a). Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. Development *122*, 2261-2269.

Burke, R. and Basler, K. (1996b). *Hedgehog*-dependent patterning in the *Drosophila* eye can occur in the absence of *Dpp* signaling. Dev. Biol. *179*, 360-368.

Campbell, G., Weaver, T., and Tomlinson, A. (1993). Axis specification in the developing *Drosophila* appendage: the role of *wingless, decapentaplegic*, and the homeobox gene *aristaless*. Cell *74*, 1113-1123.

Capdevila, J. and Guerrero, I. (1994). Targeted expression of the signaling molecule *decapentaplegic* induces pattern duplications and growth alterations in *Drosophila* wings. EMBO J. *13*, 4459-4468.

Capovilla, M., Brandt, M., and Botas, J. (1994). Direct regulation of *decapentaplegic* by *Ultrabithorax* and its role in *Drosophila* midgut morphogenesis. Cell *76*, 461-475.

Carcamo, J., Weis, F. M. B., Ventura, F., Wieser, R., Wrana, J. L., Attisano, L., and Massagué, J. (1994). Type I receptors specify growth inhibitory and transcriptional responses to TGF-β and activin. Mol. Cell Biol. *14*, 3810-3821.

Cate, R. L., Mattaliano, R. J., Hession, C., Tizard, R., Farber, N. M., Cheung, A., Ninfa, E. G., Frey, A. Z., Gash, D. J., Chow, E. P., and et al. (1986). Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells. Cell *45*, 685-698.

Chan, S.-K., Jaffe, L., Capovilla, M., Botas, J., and Mann, R. S. (1994). The DNA binding specificity of *Ultrabithorax* is modulated by cooperative interactions with *Extradenticle*, anothr homeoprotein. Cell *78*, 603-615.

Chanut, F. and Heberlein, U. (1997). Role of *decapentaplegic* in initiation and progression of the morphogenetic furrow in the developing *Drosophila* retina. Development *124*, 559-567.

Charng, M. J., Kinnunen, P., Hawker, J., Brand, T., and Schneider, M. D. (1996). FKBP-12 recognition is dispensable for signal generation by type I transforming growth factor-β receptors. J. Biol. Chem. *271*, 22941-22944.

Chen, R. H., Miettinen, P. J., Maruoka, E. M., Choy, L., and Derynck, R. A. (1995). A WD-domain protein that is associated with and phosphorylated by the type II TGF-β receptor. Nature *377*, 548-552.

Chen, X., Rubock, M. J., and Whitman, M. (1996). A transcriptional partner for MAD proteins in TGF-β signalling. Nature *383*, 691-696.

Childs, S. R. and O'Connor, M. B. (1994). Two domains of the Tolloid protein contribute to its unusual genetic interaction with *decapentaplegic*. Dev. Biol. *162*, 209-220.

Childs, S. R., Wrana, J. L., Arora, K., Attisano, L., O'Connor, M. B., and Massagué, J. (1993). Identification of a *Drosophila* activin receptor. Proc. Natl. Acad. Sci. USA *90*, 9475-9479.

Cohen, S. M. (1993). Imaginal disc development. In The Development of *Drosophila melanogaster*, M. Bate and M. Martinez Arias, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory press), pp. 747-841.

Daopin, S., Piez, K. A., Ogawa, Y., and Davies, D. R. (1992). Crystal structure of transforming growth factor-β2: An unusual fold for the superfamily. Science *257*, 369-373.

Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., and Wang, X. F. (1995). Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-dependent mechanism. Proc. Natl. Acad. Sci. USA *92*, 5545-5549.

de Celis, J. F. (1997). Expression and function of *decapentaplegic* and *thick veins* during the differentiation of the veins in the *Drosophila* wing. Development *124*, 1007-1018.

de Celis, J. F., Barrio, R., and Kafatos, F. C. (1996). A gene complex acting downstream of *dpp* in *Drosophila* wing morphogenesis. Nature *381*, 421-424.

DeRobertis, E. M. and Sasai, Y. (1996). A common plan for dorsoventral patterning in *Bilateria*. Nature *380*, 37-40.

Derynck, R. and Zhang, Y. (1996). Intracellular signalling: the mad way to do it. Curr. Biol. *6*, 1226-1229.

Diaz-Benjumea, F. J., Cohen, B., and Cohen, S. M. (1994). Cell interaction between compartments establishes the proximal-distal axis of *Drosophila* legs. Nature *372*, 175-179.

Doctor, J. S., Jackson, P. D., Rashka, K. E., Visalli, M., and Hoffmann, F. M. (1992). Sequence, biochemical characterization, and developmental expression of a new member of the TGF-β superfamily in *Drosophila melanogaster*. Dev. Biol. *151*, 491-505.

Duprez, D. M., Kostakopoulou, K., Francis-West, P. H., and Tickle, C. (1996). Activation of FGF-4 and HoxD gene expression by BMP-2 expressing cells in the developing chick limb. Development *122*, 1821-1828.

Eppert, K., Scherer, S. W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L. C., Bapat, B., Gallinger, S., Andrulis, I. L., Thomsen, G. H., Wrana, J. L., and Attisano, L. (1996). MADR2 maps to 18q21 and encodes a TGFβ-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. Cell *86*, 543-552.

Eresh, S., Riese, J., Jackson, D. B., Bohmann, D., and Bienz, M. (1997). A CREB-binding site as a target for *decapentaplegic* signalling during *Drosophila* endoderm induction. EMBO J. *16*, 2014-2022.

Ferguson, E. and Anderson, K. V. (1991). Dorsal-Ventral pattern formation in the *Drosophila* embryo: the role of zygotically active genes. Curr. Top. Dev. Biol. *25*, 17-43.

Ferguson, E. L. (1996). Conservation of dorsal-ventral patterning in arthropods and chordates. Curr. Opin. Genet. Dev. *6*, 424-431.

Ferguson, E. L. and Anderson, K. V. (1992a). *decapentaplegic* acts as a morphogen to organize dorsal-ventral pattern in the *Drosophila* embryo. Cell *71*, 451-461.

Ferguson, E. L. and Anderson, K. V. (1992b). Localized enhancement and repression of the activity of the TGF-β family member, *decapentaplegic*, is necessary for dorsal-ventral pattern formation in the *Drosophila* embryo. Development *114*, 583-597.

Fields, S. and Song, O.-K. (1989). A novel genetic system to detect protein-protein interactions. Nature *340*, 245-246.

Finelli, A. L., Bossie, C. A., Xie, T., and Padgett, R. W. (1994). Mutational analysis of the *Drosophila tolloid* gene, a human BMP-1 homolog. Development *120*, 861-870.

François, V., Solloway, M., O'Neill, J. W., Emery, J., and Bier, E. (1994). Dorsal-ventral patterning of the *Drosophila* embryo depends on a putative negative growth factor encoded by the *short gastrulation* gene. Genes Dev. *8*, 2602-2616.

Frasch, M. (1995). Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. Nature *374*, 464-467.

Fynan, T., M. and Reiss, M. (1993). Resistance to inhibition of cell growth by transforming growth factor-β and its role in oncogenesis. Critical reviews in Oncogenesis *4*, 493-540.

Gertler, F. B., Doctor, J. S., and Hoffmann, F. M. (1990). Genetic suppression of mutations in the *Drosophila abl* Proto-oncogene homolog. Science *248*, 857-860.

Glick, A. B., Lee, M. M., Darwiche, N., Kulkarni, A. B., Karlsson, S., and Yuspa, s. H. (1994). Targeted deletion of the TGF-β1 gene causes rapid progression to squamous cell carcinoma. Genes Dev. *8*, 2429-2440.

Graff, J. M., Bansal, A., and Melton, D. A. (1996). *Xenopus* Mad proteins transduce distinct subsets of signals for the TGF β superfamily. Cell *85*, 479-487.

Grieder, N. C., Nellen, D., Burke, R., Basler, K., and Affolter, M. (1995). *schnurri* is required for *Drosophila dpp* signaling and encodes a zinc finger protein similar to the mammalian transcription factor PRDII-BF1. Cell *81*, 791-800.

Griffith, D. L., Keck, P. C., Sampath, T. K., Rueger, D. C., and Carlson, W. D. (1996). Three-dimensional structure of recombinant human osterogenic protein 1: Structural paradigm for the transforming growth factor superfamily. Proc. Natl. Acad. Sci *93*, 878-883.

Grimm, S. and Pflugfelder, G. O. (1996). Control of the gene *optomotor-blind* in *Drosophila* wing development by *decapentaplegic* and *wingless*. Science *271*, 1601-4.

Gurdon, J. B., Harger, P., Mitchell, A., and Lemaire, P. (1994). Activin signalling and response to a morphogen gradient. Nature *371*, 477-492.

Hafen, E., Dickson, B., Raabe, T., D., B., Oellers, N., and van der Straten, A. (1993). Genetic analysis of the sevenless signal transduction pathway of *Drosophila*. Development *Suppl.*, 41-46.

Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science *271*, 350-353.

Hanks, A. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science *241*, 42-52.

Hannon, G. J. and Beach, D. (1994). p15INK4B is a potential effector of TGF-β induced cell cycle arrest. Nature *371*, 257-261.

Harland, R. M. (1994). The transforming growth factor β family and induction of vertebrate mesoderm: Bone morphogenetic proteins are ventral inducers. Proc. Natl. Acad. Sci. USA *91*, 10243-10246.

Hata, A., Lo, R. S., Wotton, D., Lagna, G., and Massagué, J. (1997). Mutations increasing autoinhibition inactivate tumour suppressors Samd2 and Smad4. Nature *388*, 82-87.

Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y. Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Jr., Wrana, J. L., and Falb, D. (1997). The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. Cell *89*, 1165-1173.

Hazama, M., Aono, A., Ueno, N., and Fujisawa, Y. (1995). Efficient expression of a heterodimer of bone morphogenetic protein subunits using a baculovirus expression system. Biochem. Biophys. Res. Commun. *209*, 859-866.

Heberlein, U., Wolff, T., and Rubin, G. M. (1993). The TGF-β homolog *dpp* and the segment polarity gene *hedgehog* are required for propagation of a morphogenetic wave in the *Drosophila* retina. Cell *75*, 913-926.

Held, L. I. (1995). Axes, boundaries and coordinates: The ABCs of fly leg development. BioEssays *17*, 721-732.

Hemmati-Brivanlou, A., Kelly, O., G., and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. Cell *77*, 283-295.

Heslip, T. R., Theisen, H., Walker, H., and Marsh, J. L. (1997). SHAGGY and DISHEVELLED exert opposite effects on *wingless* and *decapentaplegic* expression and on positional identity in imaginal discs. Development *124*, 1069-1078.

Hoffmann, F. M. (1991). *Drosophila abl* and genetic redundancy in signal transduction. Trends Genet. *7*, 351-355.

Hogan, B. L. (1996a). Bone morphogenetic proteins in development. Curr. Opin. Genet. Dev. *6*, 432-438.

Hogan, B. L. (1996b). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. *10*, 1580-1594.

Hogan, B. L., Blessing, M., Winnier, G. E., Suzuki, N., and Jones, C. M. (1994). Growth factors in development: the role of TGF-β related polypeptide signalling molecules in embryogenesis. Development *Suppl.*, 53-60.

Holley, S. A., Jackson, P. D., Sasai, Y., Lu, B., De Robertis, E. M., Hoffmann, F. M., and Ferguson, E. L. (1995). A conserved system for dorsal-ventral patterning in insects and vertebrates involving *sog* and chordin. Nature *376*, 249-253.

Holley, S. A., Neul, J. L., Attisano, L., Wrana, J. L., Sasai, Y., O'Connor, M. B., De Robertis, E. M., and Ferguson, E. L. (1996). The *Xenopus* dorsalizing factor noggin ventralizes *Drosophila* embryos by preventing DPP from activating its receptor. Cell *86*, 607-617.

Hoodless, P. A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M. B., Attisano, L., and Wrana, J. L. (1996). MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell *85*, 489-500.

Hursh, D. A., Padgett, R. W., and Gelbart, W. M. (1993). Cross regulation of *decapentaplegic* and *Ultrabithorax* transcription in the embryonic visceral mesoderm of *Drosophila*. Development *117*, 1211-1222.

Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., and Miyazono, K. (1997). Smad6 inhibits signalling by the TGF-β superfamily. Nature *389*, 622-626.

Immerglück, K., Lawrence, P. A., and Bienz, M. (1990). Induction across germ layers in *Drosophila* mediated by a genetic cascade. Cell *62*, 261-268.

Ingham, P. W. and Fietz, M. J. (1995). Quantitative effects of *hedgehog* and *decapentaplegic* activity on the patterning of the *Drosophila* wing. Curr. Biol. *5*, 432-440.

Irish, V. I. and Gelbart, W. M. (1987). The *decapentaplegic* gene is required for dorsal-ventral patterning of the *Drosophila* embryo. Genes Dev. *1*, 868-879.

Jackson, P. D. and Hoffmann, F. M. (1994). Embryonic expression patterns of the *Drosophila decapentaplegic* gene: separate regulatory elements control blastoderm expression and lateral ectodermal expression. Dev. Dynam. *199*, 28-44.

Jenkins, P. B., Abou-Alfa, G. K., Dhermy, D., Bursaux, E., Feo, C., Scarpa, A. L., Lux, S. E., Garbarz, M., Forget, B. G., and Gallagher, P. G. (1996). A nonsense mutation in the erythrocyte band 3 gene associated with decreased mRNA accumulation in a kindred with dominant hereditary spherocytosis. J. Clin. Invest. *97*, 373-380.

Jiang, J. and Struhl, G. (1995). Protein kinase A and *hedgehog* signaling in *Drosophila* limb development. Cell *80*, 563-572.

Jiang, J. and Struhl, G. (1996). Complementary and mutually exclusive activities of *decapentaplegic* and *wingless* organize axial patterning during *Drosophila* leg development. Cell *86*, 401-409.

Johnston, L. A. and Schubiger, G. (1996). Ectopic expression of *wingless* in imaginal discs interferes with *decapentaplegic* expression and alters cell determination. Development *122*, 3519-3529.

Kawabata, M., Imamura, T., Miyazono, K., Engel, M. E., and Moses, H. L. (1995). Interaction of the transforming factor-β type I receptor with farnesyl-protein transferase-α. J. Biol. Chem. *270*, 29628-29631.

Kessler, O. and Chasin, L. A. (1996). Effects of nonsense mutations on nuclear and cytoplasmic adenine phosphoribosyltransferase RNA. Mol. Cell Biol. *16*, 4426-4435.

Kim, J., Johnson, K., Chen, H. J., Carroll, S., and Laughon, A. (1997). *Drosophila* Mad binds to DNA and directly mediates activation of *vestigial* by Decapentaplegic. Nature *388*, 304-308.

Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J., and Carroll, S. B. (1996). Integration of positional signals and regulation of wing formation and identity by *Drosophila vestigial* gene. Nature *382*, 133-138.

Kingsley, D. M. (1994a). The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev. *8*, 133-146.

Kingsley, D. M. (1994b). What do BMPs do in mammals? Clues from the mouse *short-ear* mutation. Trends Genet. *10*, 16-21.

Kretzschmar, M., Liu, F., Hata, A., Doody, J., and Massagué, J. (1997). The TGF-β family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. Genes Dev. *11*, 984-995.

Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massague, J. (1996). Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. Nature *383*, 832-836.

Laiho, M., Weis, F. M. B., Boyd, F. T., Ignotz, R., A., and Massagué, J. (1991). Responsiveness to transforming growth factor-β restored by complementation between cells defective in TGF-β receptors I and II. J. Biol. Chem. *266*, 9108-9112.

Laiho, M., Weis, F. M. B., and Massagué, J. (1990). Concomitant loss of transforming growth factor (TGF)-β receptor types I and II in TGF-β-resistant cell mutants implicates both receptor types in signal transduction. J. Biol. Chem. *265*, 18518-18524.

Lechleider, R. J., de Caestecker, M. P., Dehejia, A., Polymeropoulos, M. H., and Roberts, A. B. (1996). Serine phosphorylation, chromosomal localization, and transforming growth factor-β signal transduction by human bsp-1. J. Biol. Chem. *271*, 17617-17620.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H., and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. Nature *381*, 387-393.

Lecuit, T. and Cohen, S. M. (1997). Proximal-distal axis formation in the *Drosophila* leg. Nature *388*, 139-145.

Lee, S. J. (1991). Expression of growth/differentiation factor 1 in the nervous system: Conservation of a bicistronic structure. Proc. Natl. Acad. Sci *88*, 4250-4254.

Lepage, T., Cohen, S. M., Diaz-Benjumea, F. J., and Parkhurst, S. M. (1995). Signal transduction by cAMP-dependent protein kinase A in *Drosophila* limb patterning. Nature *373*, 711-715.

Letsou, A., Arora, K., Wrana, J. L., Simin, K., Twombly, V., Jamal, J., Staehling-Hampton, K., Hoffmann, F. M., Gelbart, W. M., Massagué, J., and et al. (1995). *Drosophila* Dpp signaling is mediated by the *punt* gene product: a dual ligand-binding type II receptor of the TGFβ receptor family. Cell *80*, 899-908.

Li, W., Ohlmeyer, J. T., Lane, M. E., and Kalderon, D. (1995). Function of *protein kinase A* in *hedgehog* signal transduction and *Drosophila* imaginal disc development. Cell *80*, 553-62.

Lin, H. Y., Wang, X. F., Ng, E. E., Weinberg, R. A., and Lodish, H. F. (1993). GNDF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science *260*, 1130-1132.

Lindsley, D. L. and Zimm, G. G. (1992). The genome of *Drosophila melanogaster*, (New York: Academic Press).

Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and Guillemin, R. (1986). Pituitary FSH is released by a heterodimer of the β subunits from the two forms of inhibin. Nature *321*, 779-782.

Liu, F., Hata, A., Baker, J. C., Doody, J., Carcamo, J., Harland, R. M., and Massague, J. (1996). A human Mad protein acting as a BMP-regulated transcriptional activator. Nature *381*, 620-623.

Lopez, C. F., Payne, H. M., Andres, J. L., and Massagué, J. (1994). Betaglycan is a dual regulator of TGF-β access to signalling receptors. J. Cell Biol. *124*, 557-568.

Lopez, C. F., Wrana, J. L., and Massagué, J. (1993). Betaglycan presents ligand to the TGF beta signaling receptor. Cell *73*, 1435-1444.

Lyons, K. M., Hogan, B. L., and Robertson, E. J. (1995). Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development. Mech. Dev. *50*, 71-83.

Lyons, K. M., Jones, C. M., and Hogan, B. L. M. (1991). The DVR gene family in embryonic development. Trends. Gent. *7*, 408-412.

Ma, C. and Moses, K. (1995). *Wingless* and *patched* are negative regulators of the morphogenetic furrow and can affect tissue polarity in the developing *Drosophila* compound eye. Development *121*, 2279-2289.

Ma, C., Zhou, Y., Beachy, P. A., and Moses, K. (1993). The segment polarity gene *hedgehog* is required for progression of the morphogenetic furrow in the developing *Drosophila* eye. Cell *75*, 927-938.

Macias-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attisano, L., and Wrana, J. L. (1996). MADR2 is a substrate of the TGFβ receptor and its phosphorylation is required for nuclear accumulation and signaling. Cell *87*, 1215-1224.

Manak, J. R., Mathies, L. D., and Scott, M. P. (1994). Regulation of a *decapentaplegic* midgut enhancer by homeotic proteins. Development *120*, 3605-3619.
Marker, P. C., Seung, K., Bland, A. E., Russell, L. B., and Kingsley, D. M. (1997). Sepectrum of *Bmp5* mutations from germline mutagenesis experiments in mice. Genetics *145*, 435-443.

Massagué, J. (1990). The transforming growth factor-beta family. Ann. Rev. Cell Biol. *6*, 597-641.

Massagué, J. (1996). TGF-β signaling: receptors, transducers, and Mad proteins. Cell *85*, 947-950.

Massagué, J., Attisano, L., and Wrana, J. L. (1994). The TGF-β family and its composite receptors. Trends Cell Biol. *4*, 172-178.

Massagué, J. and Weis-Garcia, F. (1996). Serine/threonine kinase receptors: mediators of transforming growth factor β family signals. Cancer Surv. *27*, 41-64.

Masucci, J. D. and Hoffmann, F. M. (1993). Identification of two regions from the *Drosophila decapentaplegic* gene required for embryonic midgut development and larval viability. Dev. Biol. *159*, 276-287.

Masucci, J. D., Miltenberger, R. J., and Hoffmann, F. M. (1990). Pattern-specific expression of the *Drosophila decapentaplegic* gene in imaginal disks is regulated by 3' cis-regulatory elements. Genes Dev. *4*, 2011-2023.

McPherron, A. C. and Lee, S. J. (1993). GDF-3 and GDF-9: Two new members of the transforming growth factor-beta superfamily containing a novel pattern of cysteines. J. Biol. Chem *268*, 3444-3449.

Miyazono, K., Ichijo, H., and C-H., H. (1993). Transforming growth factor-β: latent forms, binding proteins and receptors. Growth Factors *8*, 11-22.

Miyazono, K., Ten Dijke, P., Ichijo, H., and Heldin, C. H. (1994). Receptors for transforming growth factor-β. Adv. Immunol. *55*, 181-220.

Morimura, S., Maves, L., Chen, Y., and Hoffmann, F. M. (1996). *decapentaplegic* overexpression affects *Drosophila* wing and leg imaginal disc development and *wingless* expression. Dev. Biol. *177*, 136-151.

Morisato, D. and Anderson, K. V. (1995). Signaling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. Annu. Rev. Genet. *29*, 371-399.

Moses, H. L. and Serra, R. (1996). Regulation of differentiation by TGF-β. Curr. Opin. Genet. Dev. *6*, 581-586.

Moses, H. L., Yang, E. Y., and Pietenpol, J. A. (1990). TGF-β stimulation and inhibition of cell proliferation: new mechanistic insights. Cell *63*, 245-247.

Mullor, J. L., Calleja, M., Capdevila, J., and Guerrero, I. (1997). *Hedgehog* activity, independent of *decapentaplegic*, participates in wing disc patterning. Development *124*, 1227-1237.

Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, E., Tamaki, K., Hanai, J., Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997). TGF-β receptor-mediated signalling through Smad2, Smad3 and Smad4. Development *124*, 3177-3184.

Nellen, D., Affolter, M., and Basler, K. (1994). Receptor serine/threonine kinases implicated in the control of *Drosophila* body pattern by *decapentaplegic*. Cell *78*, 225-237.

Nellen, D., Burke, R., Struhl, G., and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. Cell *85*, 357-368.

Newfeld, S. J., Chartoff, E. H., Graff, J. M., Melton, D. A., and Gelbart, W. M. (1996). *Mothers against dpp* encodes a conserved cytoplasmic protein required in DPP/TGF-β responsive cells. Development *122*, 2099-2108.

Newfeld, S. J., Mehra, A., Singer, M. A., Wrana, J. L., Attisano, L., and Gelbart, W. M. (1997). *Mothers against dpp* participates in a DPP/TGF-β responsive serine-threonine kinase signal transduction cascade. Development *124*, 3167-3176.

Niswander, L., Jeffrey, S., Martin, G. R., and Tickle, C. (1994). A positive feedback loop coordinates growth and patterning in the vertebrate limb. Nature *371*, 609-612.

Padgett, R. W., St. Johnston, R. D., and Gelbart, W. M. (1987). A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-β family. Nature 81-84.

Padgett, R. W., Wozney, J. M., and Gelbart, W. M. (1993). Human BMP sequences can confer normal dorsal-ventral patterning in the *Drosophila* embryo. Proc. Natl. Acad. Sci. USA *90*, 2905-2909.

Pan, D. and Rubin, G. M. (1995). cAMP-dependent protein kinase and *hedgehog* act antagonistically in regulating *decapentaplegic* transcription in *Drosophila* imaginal discs. Cell *80*, 543-552.

Panganiban, G. E. F., Rashka, K. E., Neitzel, M. D., and Hoffmann, F. M. (1990a). Biochemical characterization of the *Drosophila* Dpp protein, a member of the transforming growth factor-β family of growth factors. Mol. Cell Biol. *10*, 2669-2677.

Panganiban, G. E. F., Reuter, R., Scott, M. P., and Hoffmann, F. M. (1990b). A *Drosophila* growth factor homolog, *decapentaplegic*, regulates homeotic gene expression within and across germ layers during midgut morphogenesis. Development *110*, 1041-1050.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B., and Goodman, C. S. (1989). Expression of Engrailed proteins in Arthropods, Annelids and Chordates. Cell *58*, 955-968.

Patterson, G. I., Koweek, A., Wong, A., Liu, Y., and Ruvkun, G. (1997). The DAF-3 Smad protein antagonizes TGF-β-related receptor signaling in the *Caenorhabditis elegans* dauer pathway. Genes Dev. *11*, 2679-2690.

Penton, A., Chen, Y., Staehling-Hampton, K., Wrana, J. L., Attisano, L., Szidonya, J., Cassill, J. A., Massagué, J., and Hoffmann, F. M. (1994). Identification of two bone morphogenetic protein type I receptors in *Drosophila* and evidence that Brk25D is a *decapentaplegic* receptor. Cell *78*, 239-250.

Penton, A. and Hoffmann, F. M. (1996). *Decapentaplegic* restricts the domain of *wingless* during *Drosophila* limb patterning. Nature *382*, 162-164.

Penton, A., Selleck, S. B., and Hoffmann, F. M. (1997). Regulation of cell cycle synchronization by *decapentaplegic* during *Drosophila* eye development. Science *275*, 203-206.

Petraglia, F., Vaughan, J., and Vale, W. (1989). Inhibin and activin modulate the release of gonadotropin-releasing hormone, human chorionic gonadotropin and progesterone from cultured human placenta cells. Proc. Natl. Acad. Sci. USA *86*, 5114-5117.

Piccolo, S., Sasai, Y., Lu, B., and De Robertis, E. M. (1996). Dorsoventral patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP4. Cell *86*, 589-598.

Posakony, L. G., Raftery, L. A., and Gelbart, W. M. (1991). Wing formation in *Drosophila melanogaster* requires *decapentaplegic* gene function along the anterior-posterior compartment boundary. Mech. Dev. *33*, 69-82.

Raftery, L. A., Twombly, V., Wharton, K., and Gelbart, W. M. (1995). Genetic screens to identify elements of the *decapentaplegic* signaling pathway in *Drosophila*. Genetics *139*, 241-254.

Ray, R. P., Arora, K., Nüsslein-Volhard, C., and Gelbart, W. M. (1991). The control of cell fate along the dorsal-ventral axis of the *Drosophila* embryo. Development *113*, 35-54.

Reddy, K. B., Karode, M. C., Harmony, J. A. K., and Howe, P. H. (1996). Interaction of transforming growth factor β receptors with apolipoprotein J/clusterin. Biochemistry *35*, 309-314.

Reuter, R., Panganiban, G. E. F., Hoffmann, F. M., and Scott, M. P. (1990). Homeotic genes regulate the spatial expression of putative growth factors in the visceral mesoderm of *Drosophila* embryos. Development *110*, 1031-1040.

Reuter, R. and Scott, M. P. (1990). Expression and function of the homeotic genes *Antennapedia* and *Sex combs reduced* in the embryonic midgut of *Drosophila*. Development *109*, 289-303.

Reynisdottir, I., Polyak, K., Iavarone, A., and Massagué, J. (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β. Genes Dev. *9*, 1831-1845.

Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S. C., Grosschedl, R., and Bienz, M. (1997). LEF-1, a nuclear factor coordinating signaling inputs from *wingless* and *decapentaplegic*. Cell *88*, 777-787.

Roberts, A. B., A., A. M., Lamb, L. C., Smith, J. M., and Sporn, M. B. (1981). New class of transforming growth factors potentiated by epidermal growth factor. Proc. Natl. Acad. Sci. USA *78*, 5339-5343.

Roberts, A. B. and Sporn, M. B. (1990). The transforming growth factor-betas. In Peptide growth factors and their receptors, M. B. Sporn and A. B. Roberts, ed. (Heidelberg: Springer-Verlag), pp. 419-472.

Roberts, D. J., Johnson, R. L., Burke, A. C., Nelson, C. E., Morgan, B. A., and Tabin, C. (1995). Sonic *hedgehog* is an endodermal signal inducing *Bmp-4* and *Hox* genes during induction and regionalization of the chick hindgut. Development *121*, 3163-3174.

Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D. M., Benz, W. K., and Engels, W. R. (1988). A stable source of P element transposase in *Drosophila melanogaster*. Genetics *118*, 461-470.

Ruberte, E., Narty, T., Nellen, D., Affolter, M., and Basler, K. (1995). An absolute requirement for both the type II and type I receptors, *Punt* and *thickveins*, for *dpp* signaling *in vivo*. Cell *80*, 889-897.

Rusch, J. and Levine, M. (1996). Threshold responses to the dorsal regulatory gradient and the subdivision of primary tissue territories in the *Drosophila* embryo. Curr. Opin. Genet. Dev. *6*, 416-423.

Sambrook, J., Fritsch, E., F., and Maniatis, T. (1989). Molecular cloning: A laboratory manual, (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sampath, T. K., Rashka, K. E., Doctor, J. S., Tucker, R. F., and Hoffmann, F. M. (1993). *Drosophila* TGF-β superfamily proteins induce endochondral bone formation in mammals. Proc. Natl. Acad. Sci. USA *90*, 6004-6008.

Savage, C., Das, P., Finelli, A. L., Townsend, S. R., Sun, C. Y., Baird, S. E., and Padgett, R. W. (1996). *Caenorhabditis elegans* genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor β pathway components. Proc. Natl. Acad. Sci. USA *93*, 790-794.

Schmidt, J., Francois, V., Bier, E., and Kimelman, D. (1995). *Drosophila short gastrulation* induces an ectopic axis in *Xenopus*: evidence for conserved mechanisms of dorsal-ventral patterning. Development *121*, 4319-4328.

Schutte, M., Hruban, R. H., Hedrick, L., Cho, K. R., Nadasdy, G. M., Weinstein, C. L., Bova, G. S., Isaacs, W. B., Cairns, P., Nawroz, H., Sidransky, D., Casero, R. A., Jr., Meltzer, P. S., Hahn, S. A., and Kern, S. E. (1996). DPC4 gene in various tumor types. Cancer Res. *56*, 2527-2530.

Sekelsky, J., Newfeld, S. J., Raftery, L. A., Chartoff, E. H., and Gelbart, W. M. (1995). Genetic characterization and cloning of *mothers against dpp*, a gene required for *decapentaplegic* function in *Drosophila melanogaster*. Genetics *139*, 1347-1358.

Sherr, C. J. and Roberts, J. M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. *9*, 1149-1163.

Shi, Y., Hata, A., Lo, R. S., Massagué, J., and Pavletich, N. P. (1997). A structural basis for mutational inactivation of the tumour suppressor Smad4. Nature *388*, 87-93.

Shibuya, H., Yamaguchi, K., Shirakabe, K., Tonegawa, A., Gotoh, Y., Ueno, N., Irie, K., Nishida, E., and Matsumoto, K. (1996). TAB1: an activator of the TAK1 MAPKKK in TGF-β signal transduction. Science *272*, 1179-1182.

Shimell, M. J., Ferguson, E. R., Childs, S. R., and O'Connor, M. B. (1991). The *Drosophila* dorsal-ventral patterning gene *tolloid* is related to human bone morphogenetic protein 1. Cell *67*, 469-481.

Simon, M. A., Bowtell, D. D. L., Dodson, G. S., Laverty, T. R., and Rubin, G. M. (1991). Ras 1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the Sevenless protein tyrosine kinase. Cell *67*, 701-716.

Singer, M. A., Penton, A., Twombly, V., Hoffmann, F. M., and Gelbart, W. M. (1997). Signaling through both type I DPP receptors is required for anterior- posterior patterning of the entire *Drosophila* wing. Development *124*, 79-89.

Spencer, F. A., Hoffmann, F. M., and Gelbart, W. M. (1982). *Decapentaplegic*: A gene complex affecting morphogenesis in *Drosophila melanogaster*. Cell *28*, 451-461.

Spradling, A. C. and Rubin, R. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. Science *218*, 341-347.

St. Johnston, R. D. and Gelbart, W. D. (1987). *Decapentaplegic* transcripts are localized along the dorsal-ventral axis of the *Drosophila* embryo. EMBO J. *6*, 2785-2791.

St. Johnston, R. D., Hoffmann, F. M., Blackman, R. K., Segal, D., Grimaila, R., Padgett, R. W., Irick, H. A., and Gelbart, W. M. (1990). The molecular organization of the *decapentaplegic* gene in *Drosophila melanogaster*. Genes Dev. 4, 1114-1127.

Staehling-Hampton, K. and Hoffmann, F. M. (1994). Ectopic *decapentaplegic* in the *Drosophila* midgut alters the expression of five homeotic genes, *dpp* and *wingless*, causing morphological defects. Dev. Biol. *164*, 502-512.

Staehling-Hampton, K., Hoffmann, F. M., Baylies, M. K., Rushton, E., and Bate, M. (1994). *dpp* induces mesodermal gene expression in *Drosophila*. Nature *372*, 783-786.

Staehling-Hampton, K., Laughon, A. S., and Hoffmann, F. M. (1995). A *Drosophila* protein related to the human zinc finger transcription factor PRDII/MBPI/HIV-EP1 is required for *dpp* signaling. Development *121*, 3393-3403.

Struhl, G. (1989). Differing strategies for organizing anterior and posterior body pattern in *Drosophila* embryos. Nature *338*, 741-744.

Struhl, G. and Basler, K. (1993). Organizing activity of Wingless protein in *Drosophila*. Cell *72*, 527-540.

Sun, B., Hursh, D. A., Jackson, D., and Beachy, P. A. (1995). *Ultrabithorax* protein is necessary but not sufficient for full activation of *decapentaplegic* expression in the visceral mesoderm. EMBO J. *14*, 520-535.

Suzuki, A., Kaneko, E., Maeda, J., and Ueno, N. (1997). Mesoderm induction by BMP-4 and -7 heterodimers. Biochem. Biophys. Res. Commun. *232*, 153-156.

Szidonya, J. and Reuter, G. (1988a). Cytogenetic analysis of the *ecinoid* (*ed*), *dumpy* (*dp*) and *clot* (*cl*) region in *Drosophila melanogaster*. Genet. Res., Camb. *51*, 197-208.

Szidonya, J. and Reuter, G. (1988b). Cytogenetics of the 24D4-25F2 region of the *Drosophila melanogaster* 2L chromosome. *Drosophila* Information Service *67*, 77-79.

Theisen, H., Haerry, T. E., O'Connor, M. B., and Marsh, J. L. (1996). Developmental territories created by mutual antagonism between Wingless and Decapentaplegic. Development *122*, 3939-3948.

Thiagalingam, S., Lengauer, C., Leach, F. S., Schutte, M., Hahn, S. A., Overhauser, J., Willson, J. K., Markowitz, S., Hamilton, S. R., Kern, S. E., Kinzler, K. W., and Vogelstein, B. (1996). Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. Nat. Genet. *13*, 343-346.

Thomsen, G. H. (1996). *Xenopus* mothers against decapentaplegic is an embryonic ventralizing agent that acts downstream of the BMP-2/4 receptor. Development *122*, 2359-2366.

Thüringer, F., Cohen, S. M., and Bienz, M. (1993a). Dissection of an indirect autoregulatory response of a homeotic *Drosophila* gene. EMBO J. *12*, 2419-2430.

Thüringer, F., Cohen, S. M., and Bienz, M. (1993b). Indirect autoregulation of a homeotic *Drosophila* gene mediated by extracellular signaling. Proc. Natl. Acad. Sci. USA *90*, 3899-3903.

Tomlinson, A. and Ready, D. F. (1987). Neuronal differentiation in the *Drosophila* ommatidium. Dev. Biol. *120*, 366-376.

Treisman, J. E. and Rubin, G. M. (1995). *wingless* inhibits morphogenetic furrow movement in the *Drosophila* eye disc. Development *121*, 3519-3527.

Tremml, G. and Bienz, M. (1989). Homeotic gene expression in the visceral mesoderm of *Drosophila* embryos. EMBO J. *8*, 2677-2685.

Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Kornberg, T. B., Christian, J. L., and Tabata, T. (1997). *Daughters against dpp* modulates *dpp* organizing activity in *Drosophila* wing development. Nature *389*, 627-631.

Twombly, V., Blackman, R. K., Jin, H., Graff, J. M., Padgett, R. W., and Gelbart, W. M. (1996). The TGF-β signaling pathway is essential for *Drosophila* oogenesis. Development *122*, 1555-1565.

Vaahtokari, A., Abert, T., Jernvall, J., Keranen, S., and Thesleff, I. (1996). The enamel knot as a signaling center in the developing mouse tooth. Mech. Dev. *54*, 39-43.

van Hoof, A. and Green, P. J. (1996). Premature nonsense codons decrease the stability of phytohemagglutinin mRNA in a position-dependent manner. Plant J. *10*, 415-424.

Ventura, F., Liu, F., Doody, J., and J., M. (1996). Transforming growth factor-β receptor type I interacts with farnesyl transferase-α. J. Biol. Chem. *271*, 13931-13934.

Wang, T., Danielson, P. D., Li, B., Shah, P. C., Kim, S. D., and Donahoe, P. K. (1996a). The p21Ras farnesyltransferase-α subunit in TGFβ and activin signaling. Science *271*, 1120-1122.

Wang, T., Donahoe, P. K., and Zervos, A. S. (1994). Specific interaction of type I receptors of the TGF-β family with the immunophilin FKBP-12. Science *265*, 674-676.

Wang, T., Li, B.-Y., Danielson, P. D., Rockwell, S., Lechleider, R. J., Martin, J., Manganaro, T., and Donahoe, P. K. (1996b). The immunophilin FKBP12 functions as a common inhibitor of the TGFβ family type I receptors. Cell *86*, 435-444.

Wharton, K. A., Ray, R. P., and Gelbart, W. M. (1993). An activity gradient of *decapentaplegic* is necessary for the specification of dorsal pattern elements in the *Drosophila* embryo. Development *117*, 807-822.

Wharton, K. A., Thomsen, G. H., and Gelbart, W. M. (1991). *Drosophila* 60A gene, a new TGF-ß family member is closely related to human bone morphogenetic proteins. Proc. Natl. Acad. Sci. USA *88*, 9214-9218.

Wiersdorff, V., Lecuit, T., Cohen, S. M., and Mlodzik, M. (1996). *Mad* acts downstream of Dpp receptors, revealing a differential requirement for *dpp* signaling in initiation and propagation of morphogenesis in the *Drosophila* eye. Development *122*, 2153-2162.

Williams, J. A., Paddock, S. W., Vorwerk, K., and Carroll, S. B. (1994). Organization of wing formation and induction of a wing-patterning gene at the dorsal/ventral compartment boundary. Nature *368*, 299-305.

Wilson, P., Lagna, G., Suzuki, A., and Hemmati-Brivanlou, A. (1997). Concentration-dependent patterning of the *Xenopus* ectoderm by BMP4 and its signal transducer Smad1. Development *124*, 3177-3184.

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988). Novel regulators of bone formation: Molecular clones and activities. Science *242*, 1528-1534.

Wrana, J. L. and Attisano, L. (1996). MAD-related proteins in TGFβ signaling. Trends Genet. *12*, 493-496.

Wrana, J. L., Attisano, L., Cárcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.-F., and Massagué, J. (1992). TGF-β signals through a heteromeric protein kinase receptor complex. Cell *71*, 1003-1014.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massagué, J. (1994). Mechanism of activation of the TGF-β receptor. Nature *370*, 341-347.

Wrana, J. L. and Pawson, T. (1997). Mad about SMADs. Nature *388*, 28-29.

Wu, R. Y., Zhang, Y., Feng, X. H., and Derynck, R. (1997). Heteromeric and homomeric interactions correlate with signaling activity and functional cooperativity of Smad3 and Smad4/DPC4. Mol. Cell Biol. *17*, 2521-2128.

Xie, T., Finelli, A. L., and Padgett, R. W. (1994). The *Drosophila saxophone* gene: a serine-threonine kinase receptor of the TGF-β superfamily. Science *263*, 1756-1759.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development *117*, 1223-1237.

Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995). Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. Science *270*, 2008-2011.

Yamashita, H., ten Dijke, P., Huylebroeck, D., Sampath, T. K., Andries, M., Smith, J. C., Heldin, C.-H., and Miyazono, K. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. J. Cell Biol. *130*, 217-226.

Ying, S. Y. (1989). Inhibins, activins and follistatins. J. Steroid Biochem. *33*, 705-713.

Yingling, J. M., Das, P., Savage, C., Zhang, M., Padgett, R. W., and Wang, X. F. (1996). Mammalian dwarfins are phosphorylated in response to transforming growth factor β and are implicated in control of cell growth. Proc. Natl. Acad. Sci. USA *93*, 8940-8944.

Yu, X., Hoppler, S., Eresh, S., and Bienz, M. (1996). *decapentaplegic*, a target gene of the *wingless* signalling pathway in the *Drosophila* midgut. Development *122*, 849-858.

Zecca, M., Basler, K., and Struhl, G. (1995). Sequential organizing activities of *engrailed, hedgehog* and *decapentaplegic* in the *Drosophila* wing. Development *121*, 2265-2278.

Zhang, Y., Feng, X., We, R., and Derynck, R. (1996). Receptor-associated Mad homologues synergize as effectors of the TGF-β response. Nature *383*, 168-172.

Zhang, Y., Musci, T., and Derynck, R. (1997). The tumor suppressor Smad4/DPC 4 as a central mediator of Smad function. Curr. Biol. *7*, 270-276.

Development 125, 1759-1768 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV5183



# A genetic screen for modifiers of *Drosophila decapentaplegic* signaling identifies mutations in *punt, Mothers against dpp* and the BMP-7 homologue, *60A*

**Yijing Chen[1], Matthew J. Riese[1], Mark A. Killinger[1] and F. Michael Hoffmann[1,2,*]**

[1]McArdle Laboratory for Cancer Research and [2]Laboratory of Genetics, Medical School, University of Wisconsin-Madison, Madison, Wisconsin, 53706, USA
*Author for correspondence (e-mail: fmhoffma@facstaff.wisc.edu)

*Accepted 26 February; published on WWW 1 April 1998*

## SUMMARY

***decapentaplegic (dpp)* is a Transforming Growth Factor beta (TGF-β)-related growth factor that controls multiple developmental processes in *Drosophila*. To identify components involved in *dpp* signaling, we carried out a genetic screen for dominant enhancer mutations of a hypomorphic allele of *thick veins (tkv)*, a type I receptor for *dpp*. We recovered new alleles of *tkv, punt, Mothers against dpp (Mad)* and *Medea (Med)*, all of which are known to mediate *dpp* signaling. We also recovered mutations in the *60A* gene which encodes another TGF-β-related factor in *Drosophila*. DNA sequence analysis established that all three *60A* alleles were nonsense mutations in the prodomain of the *60A* polypeptide. These mutations in *60A* caused defects in midgut morphogenesis and fat body differentiation. We present evidence that when *dpp* signaling is compromised, lowering the level of *60A* impairs several *dpp*-dependent developmental processes examined, including the patterning of the visceral mesoderm, the embryonic ectoderm and the imaginal discs. These results provide the first in vivo evidence for the involvement of *60A* in the *dpp* pathway. We propose that *60A* activity is required to maintain optimal signaling capacity of the *dpp* pathway, possibly by forming biologically active heterodimers with Dpp proteins.**

Key words: *dpp, punt, Mad, 60A*, Modifier screen, TGF-β, *Drosophila*

## INTRODUCTION

The Transforming Growth Factor-beta (TGF-β) superfamily is a family of conserved polypeptide growth factors that regulate diverse biological activities (reviewed by Kingsley, 1994a; Massagué, 1996). In particular, within the TGF-β superfamily, the Bone Morphogenetic Proteins (BMPs) are critical regulators of cell proliferation, cell death, cell fate specification and organogenesis (reviewed by Kingsley, 1994b; Hogan, 1996). The versatile signaling capacity of TGF-β-related factors partially stems from extensive post-translational modifications that occur during the maturation process. These factors are synthesized as large precursor molecules, which undergo homomeric or heteromeric dimerization and subsequent proteolytic cleavage to yield the bioactive carboxy terminal portion (Roberts and Sporn, 1990). Upon secretion, their association with extracellular binding proteins is important for regulating their access to cell surface receptors (reviewed by Miyazono et al., 1993). The interaction with a heteromeric transmembrane receptor system composed of two distinct serine/threonine kinases (type I and II receptors) adds one more level of complexity to TGF-β-related signaling (Massagué and Weis-Garcia, 1996). In order to understand the complex processes involved in TGF-β signaling, we focus on *decapentaplegic* (*dpp*), a *Drosophila* gene that is functionally interchangeable with mammalian BMP-2 and BMP-4 (Padgett et al., 1993; Sampath et al., 1993).

*dpp* has a dynamic expression pattern and multiple functions throughout *Drosophila* development. At the blastoderm stage, *dpp* transcripts are restricted dorsally (St. Johnston and Gelbart, 1987) where it functions as a morphogen to specify distinct dorsal structures (Ferguson and Anderson, 1992a,b; Wharton et al., 1993). Later during embryogenesis, *dpp* is expressed in the ectoderm (Jackson and Hoffmann, 1994), where it induces dorsal mesoderm differentiation (Staehling-Hampton et al., 1994). In the visceral mesoderm, *dpp* is expressed in discrete domains (Panganiban et al., 1990b) to regulate the expression of several homeotic genes in different tissue layers (Bienz, 1994). *dpp* is also expressed in specific positions in the larval imaginal discs (Masucci et al., 1990) to control the proliferation and patterning of adult appendages (reviewed by Brook et al., 1996).

In addition to *dpp*, *60A* and *scw* are two other BMP-related genes in *Drosophila* (Arora et al., 1994; Doctor et al., 1992; Wharton et al., 1991). Scw proteins have been proposed to enhance *dpp* activity during early embryogenesis by forming heterodimers with Dpp (Arora et al., 1994). The role of *60A* in

development was unclear due to the lack of knowledge of the phenotypic consequences of disrupting *60A* function.

In *Drosophila*, two type I receptors encoded by *saxophone* (*sax*) and *thick veins* (*tkv*) and one type II receptor encoded by *punt* have been shown to be functional *dpp* receptors (Penton et al., 1994; Brummel et al., 1994; Nellen et al., 1994; Ruberte et al., 1995; Letsou et al., 1995; Xie et al., 1994). *Mothers against dpp* (*Mad*), *Medea* (*Med*) and *schnurri* (*shn*) were identified through genetic interactions with *dpp* (Raftery et al., 1995; Sekelsky et al., 1995; Staehling-Hampton et al., 1995; Grieder et al., 1995; Arora et al., 1995). *shn* encodes a protein related to human zinc finger transcription factor PRDII/MBPI/HIV-EP1 (Staehling-Hampton et al., 1995; Arora et al., 1995; Grieder et al., 1995). *Mad*-related proteins (Smads) have been isolated from a wide range of distantly related organisms. Genetic and biochemical evidence indicated that Smads are key signal transducers, linking events between receptor activation and changes in target gene expression (Derynck and Zhang, 1996; Massagué, 1996; Wrana and Pawson, 1997).

The dosage-sensitive nature of *dpp* signaling prompted us to use modifier genetics to identify additional components in the *dpp* pathway. This approach exploits synergistic interactions between components in the same biological pathway. We sensitized the *dpp* pathway using a hypomorphic *dpp* receptor, $tkv^6$, which had a mild visible phenotype. We reasoned that, in this genetic background where *dpp* signaling is below optimal level, a two-fold reduction in the activities of other signaling components in the pathway would produce a modified phenotype. So we screened for mutations that dominantly modified $tkv^6$ phenotype. Such an approach has successfully identified components in several *Drosophila* signal transduction pathways, including the *sevenless* receptor tyrosine kinase pathway (Simon et al., 1991), the *Abelson* cytoplasmic tyrosine kinase pathway (Gertler et al., 1990) and the *dpp* pathway (Raftery et al., 1995).

In our screen, we recovered new alleles of *tkv*, *punt*, *Mad* and *Med*; all are known to mediate *dpp* signaling. Two other complementation groups were identified that potentially represent new components in the pathway. Most significantly, mutations in *60A*, the *Drosophila* homologue of BMP-7, were recovered as enhancers for the sensitized *dpp* pathway. We describe the loss-of-function phenotypes of *60A* and present the first in vivo evidence that *60A* acts synergistically with *dpp* in several developmental processes.

## MATERIALS AND METHODS

### *Drosophila* stocks

*Drosophila* stocks were cultured on standard cornmeal yeast extract sugar medium at 25°C. *Canton S.* was used as the wild-type stock. $sax^5$ was described in Twombly et al. (1996). $Mad^P$ is described in Sekelsky et al. (1995). $Med^4$ was described in Raftery et al. (1995). All other mutants and chromosomes are referenced in Flybase (http://cbbridges.harvard.edu).

### Isolation and analysis of enhancer mutations of $tkv^6$

The initial attempts to recover modifiers of $tkv^6$ in an $F_1$ screen was not successful because of greatly reduced viability and fertility of the flies with enhanced phenotypes. Thus an $F_2$ screen was carried out.

*pr cn* was recombined onto the $tkv^6$ chromosome and the stock was made isogenic for the second and the third chromosomes. $tkv^6$ *pr cn/CyO* males were mutagenized with 3.4 mM ethylnitrosourea (ENU) and mated to females with a translocation between *CyO* and *TM6,B* to force the co-segregation of the second and third chromosome. Individual male progeny were mated to $tkv^6$*/CyO; TM2/TM6,B* females. Enhancement of the phenotypes of the imaginal-disc-derived structures were screened in the progeny homozygous for $tkv^6$. The enhancer mutations were recovered from the siblings of the enhanced progeny and crossed to $tkv^6$*/CyO; TM2/TM6,B* females for retesting and to establish balanced stocks.

The number of complementation groups was determined from inter se crosses among enhancers. For enhancers on the second chromosome, a *tkv* transgene on the third chromosome (Y. C. and F. M. H., unpublished data) was used to compensate for the $tkv^6$ mutation present on the second chromosome. Allelism with known mutations was established by genetic non-complementation and by meiotic and deletion mapping. The *Sp Bl* $L^{rm}$ *Bc* $Pu^2$ $Pin^B$ chromosome was used for meiotically mapping enhancers on the second chromosome.

### Sequencing of mutant alleles

Total RNAs were isolated from heterozygous *Mad* and *punt* females and heterozygous *60A* males using the Tri Reagent (Molecular Research Center, Inc.). Gene-specific cDNAs were reverse transcribed and amplified using the Superscript Preamplification System (GIBCO BRL). The PCR products were subcloned into TA cloning vectors (Invitrogen) and sequenced on an automated sequencer (ABI 373). Sequencing multiple alleles of the same gene allowed identification of the polymorphisms specific to the mutagenized chromosomes. For $60A^{D4}$ and $60A^{D8}$, the genomic region of *60A* was sequenced in a similar fashion because the mutant cDNAs were under-represented.

### Preparation of larval gut, cuticle and adult appendages

Larval gut was dissected and mounted according to Masucci and Hoffmann (1993). Cuticles were prepared as described previously (Struhl, 1989). Wings and legs were mounted in Gary's magic mounting media (Ashburner, 1989).

### Antibody staining and identification of mutant embryos

Anti-Scr, Anti-Ubx, Anti-Lab antibodies were gifts of Dr Matthew Scott, Stanford University. Anti-Wg antibody was a gift of Dr Roel Nusse, Stanford University. Anti-Dpp antibody was described in Panganiban et al. (1990a). Antibody stainings were done as previously described (Panganiban et al., 1990b; Staehling-Hampton and Hoffmann, 1994; Reuter et al., 1990). All stocks used for antibody staining were balanced over a *CyO* chromosome with an *elav-lacZ* enhancer trap to allow unambiguous identification of mutant embryos.

## RESULTS

### *shn* and *punt* enhance $tkv^6$ phenotypes

$tkv^6$ is a mutation in a splice acceptor site that results in aberrant in-frame splicing, deleting two extracellular amino acids of the receptor. When expressed in COS1 cells, the mutant receptor fails to bind BMP-2 homodimers (Penton et al., 1994). However, $tkv^6$ behaves genetically as a hypomorph. In contrast to the embryonic lethal *tkv* null alleles, $tkv^6$ is homozygous viable and the only visible phenotype is the thickened wing veins (Fig. 1B). All other imaginal-disc-derived structures of $tkv^6$ homozygotes appear normal (Figs 1C, 3A,C). Interestingly, $tkv^6$*/Df(2L)tkv2* flies are phenotypically identical to $tkv^6$ homozygotes (data not shown). To test if $tkv^6$ is a suitable genetic background for a modifier screen, we examined the effects of lowering the activity of other known *dpp* pathway components. We found that

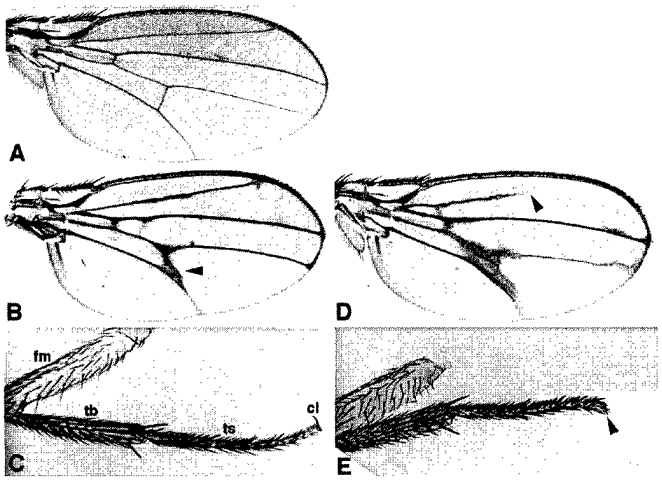



**Fig. 1.** *shn* enhanced $tkv^6$ homozygous phenotypes. (A) Wild-type wing. (B) $tkv^6$ wing. Thickening of the cross veins and the terminals of the longitudinal veins were evident (arrowhead). (D) $tkv^6 shn^{IB}/tkv^6$ wing. Besides the thickened wing veins, the longitudinal vein 2 was truncated (arrowhead). (C) Phenotypically normal $tkv^6$ mesothoracic leg. (E) $tkv^6 shn^{IB}/tkv^6$ mesothoracic leg. The distal tarsal segments and the claws were missing (arrowhead). fm, femur; tb, tibia; ts, tarsal segments; cl, claws.

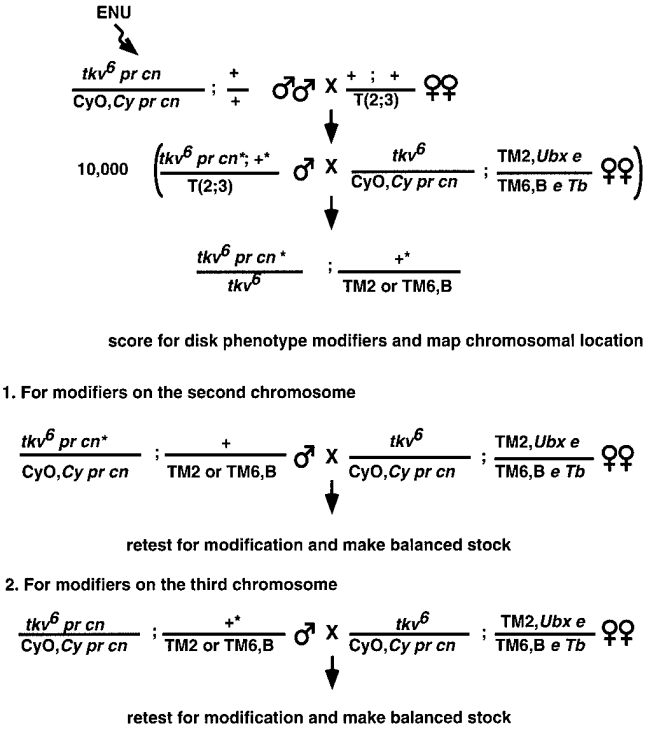



**Fig. 2.** Scheme for the $F_2$ enhancer screen of $tkv^6$. See Materials and Methods for detailed description of the procedure. ENU, Ethylnitrosourea; T(2;3), a translocation between *CyO* and *TM6,B*. Asterisks indicate the mutagenized chromosomes.

heterozygous mutations in *shn* or *punt* enhanced the $tkv^6$ homozygous phenotype. In the $tkv^6$ background, $shn^{IB}$ was a dominant enhancer of the venation pattern in the wing (Fig. 1D) and the proximal/distal patterning of the leg (Fig. 1E). In the wing, longitudinal vein 2 failed to reach the wing margin (Fig. 1D). In the leg, distal elements such as claws and distal tarsal segments were deleted (Fig. 1E). Such phenotypes were reminiscent of hypomorphic *dpp* phenotypes (Spencer et al., 1982). $punt^{135}$ also enhanced the $tkv^6$ phenotypes (data not shown). Based on these observations, we reasoned that the *dpp* signaling output through the mutant receptor $tkv^6$ was near the threshold for proper patterning of the imaginal discs. It was therefore an appropriate genetic background for identifying new components essential for mediating *dpp* signaling.

## Enhancers of *tkv* are phenotypically similar to *dpp* mutants

The modifier screen was conducted as outlined in Fig. 2. The $F_2$ progeny were screened for enhanced phenotypes in the imaginal-disc-derived structures. Over 10,000 mutagenized genomes were screened and fourteen dominant enhancers defining seven loci were recovered (Table 1). The enhancers were recessive lethal in a wild-type background. $tkv^6$ homozygotes that are heterozygous for the enhancer mutations had defects in imaginal disc development (Fig. 3). During pupal development, the dorsal proximal region of the two wing imaginal discs fuse to form the adult notum. In $tkv^6$ flies, the notum appeared normal with a smooth contour and orderly oriented sensory bristles (Fig. 3A). This pattern was disrupted by heterozygous *D1* mutation, resulting in a medial cleft in the notum, with abnormally parted bristles on both sides of the cleft (Fig. 3B). $tkv^6$ flies had normally patterned legs (Fig. 3C). However, heterozygous *D4* mutation caused deletions of distal and dorsal structures (Fig. 3D,E) and occasional duplication of ventrolateral structures such as sex combs on male prothoracic legs (Fig. 3E). These phenotypes were indistinguishable from

**Table 1. Summary of enhancer mutations**

| Complementation group | Enhancement | Chromosomal location | Number of alleles | Allelic to |
|---|---|---|---|---|
| *D1* | notum | second | 1 | – |
| *D2* | notum | second | 1 | – |
| *D3* | legs and notum | second | 5 (*D3, D14, D15, D16, D24*) | *Mad* |
| *D17* | legs and notum | second | 1 | *tkv* |
| *D4* | legs and notum | second | 3 (*D4, D8, D20*) | *60A* |
| *D5* | legs and notum | third | 1 | *Med* |
| *D13* | legs and notum | third | 2 (*D13, D18*) | *punt* |

The assignment of the enhancer mutations to different complementation groups is based on genetic mapping and inter se complementation tests among enhancer mutations as described in Materials and Methods.

**Table 2. Heterozygous interactions between enhancer mutations and *dpp* pathway mutations**

| | $tkv^6$ | *Df(2L)tkv2* | $dpp^{s5}$ | $shn^{IB}$ | $sax^5$ | $Mad^P$ | $Med^4$ | $punt^{135}$ |
|---|---|---|---|---|---|---|---|---|
| $tkv^6$ | –# | –# | – | – | – | – | – | – |
| $tkv^6D1$ | 67 | 71 | 64 | 60 | – | – | 31 | 40 |
| $tkv^6D2$ | 43 | 53 | 34 | – | 31 | – | - | 41 |
| $tkv^6D3$ | 94* | 100* | 96 | 100* | – | lethal | 23 | 68 |
| $tkv^6D15$ | 96* | 91* | 100 | 100* | – | lethal | 29 | 78 |
| $tkv^6D24$ | 100* | 100* | 100 | 100* | – | lethal | 41 | 81 |
| $tkv^6D14$ | 98 | lethal | 68 | – | 44 | lethal | – | 35 |
| $tkv^6D16$ | 31 | 50 | – | – | 41 | 48 | – | – |
| $tkv^6D17$ | 65 | lethal | – | 15 | 68 | – | – | – |
| $tkv^6D4$ | 78 | 83 | 37 | 43 | – | – | – | 25 |
| $tkv^6D8$ | 82 | 87 | 31 | 39 | – | – | – | 22 |
| $tkv^6D20$ | 68 | 73 | 25 | 50 | – | – | – | 31 |
| *D5* | 47 | 52 | ND | ND | ND | ND | lethal | – |
| *D13* | 61 | 63 | ND | ND | ND | ND | – | lethal |
| *D18* | 62 | 67 | ND | ND | ND | ND | – | lethal |

Numbers represent the percentage of heterozygous progeny with disk phenotypes such as gaps in wing veins, cleft in the notum and/or distal truncation of the legs.

lethal, failure to recover the indicated progeny class.

*Severe reduction of the indicated progeny class (less than 10%).

Minus, no interactions observed.

ND, not done.

#Thickened wing veins only, due to non-complementation with *tkv* alleles.

those of $dpp^{disk}$ alleles (Spencer et al., 1982), suggesting that these enhancers act in the *dpp* signal transduction pathway.

Additional evidence that the enhancers mediate *dpp* signaling came from their dosage-sensitive interactions with known mutations in the *dpp* pathway, including $dpp^{s5}$, $tkv^6$, *Df(2L)tkv2*, $sax^5$, $punt^{135}$, $Mad^P$, $Med^4$ and $shn^{IB}$ (Table 2). In many cases, the enhancers failed to fully complement these mutations and showed imaginal disc development defects ranging from gaps in wing veins to the notum and leg phenotypes described in Fig. 3. $tkv^6$ alone showed no detectable heterozygous interactions with the *dpp* pathway mutations examined except for the thickened venation phenotype when in *trans* to $tkv^6$ or *Df(2R)tkv2,* indicating that phenotypes observed were due to the presence of the enhancer mutations.

## Dominant enhancers of $tkv^6$: new alleles of *tkv, Mad, Med, punt* and *60A*

Meiotic mapping and complementation tests established seven complementation groups for the enhancers. As expected based on the initial evaluation of the $tkv^6$ genetic background, new alleles of *tkv*, *Mad*, *Med* and *punt* were recovered (Table 1). We sequenced the coding regions of the new *Mad* and *punt* alleles to establish their molecular identity. Of the five *Mad* alleles, three had point mutations in the coding region (Fig. 4A). Mis-sense mutations were found in both new *punt* alleles (Fig. 4B). The $tkv^{D17}$ and $Med^{D5}$ allelism was based on genetic non-complementation. In addition, it was found that a *tkv* transgene rescued the lethality of $tkv^{D17}$ homozygotes, supporting the view that *D17* is a *tkv* allele (data not shown).

Genetic and molecular characterizations of the *D4* complementation group revealed that it corresponded to the *60A* gene. *60A* encodes a BMP-7 homologue isolated based on sequence homology (Doctor et al., 1992; Wharton et al., 1991). Its function remained unknown due to the lack of mutations in *60A*. Three alleles of *60A* were confirmed by sequencing the mutant alleles. $60A^{D8}$ and $60A^{D20}$ are nonsense mutations in the prodomain due to single nucleotide substitutions. $60A^{D4}$ has one nucleotide deletion, causing a frame-shift premature stop also in the prodomain (Fig. 4C).

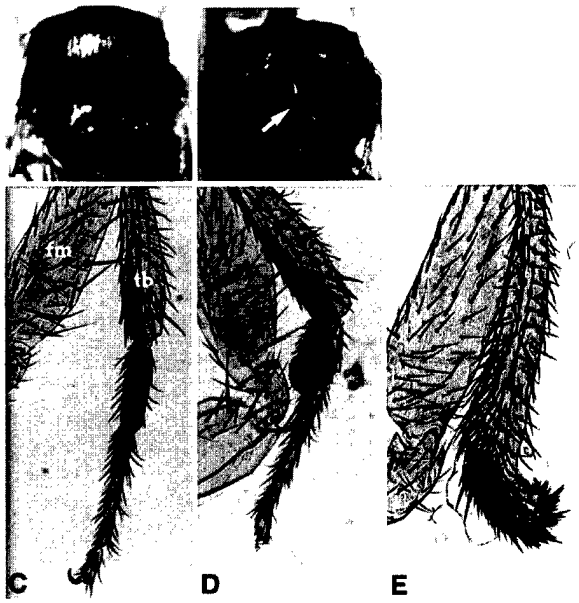



**Fig. 3.** Heterozygous enhancer mutations enhanced $tkv^6$ phenotypes. (A) Phenotypically normal $tkv^6$ notum. (B) $tkv^6D1/tkv^6$ notum. The sensory bristles were parted to both sides and there was a profound medial cleft (arrow). The scutellum was often reduced in size (compare with A). (C) Phenotypically normal $tkv^6$ male prothoracic leg. (D) $tkv^660A^{D4}/tkv^6$ male prothoracic leg. The most distal tarsal segments and claws were truncated (arrow) and the ventral lateral sex combs were duplicated (arrow head). (E) $tkv^660A^{D4}/tkv6$ metathoracic leg. Note the severe truncation of distal structures and the curved appearance of the leg caused by the loss of dorsal tissues (arrow). fm, femur; tb, tibia; sc, sex combs; ts, tarsal segments; cl, claws.

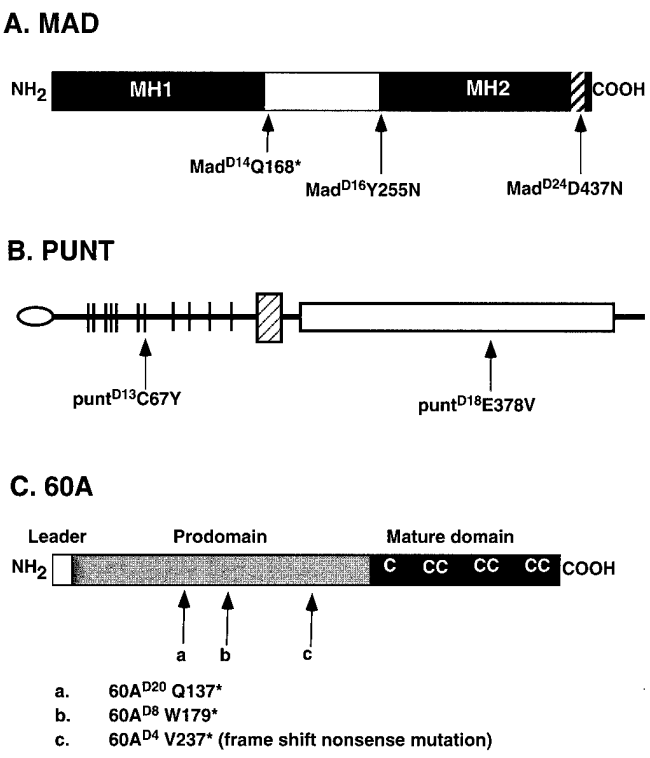



**Fig. 4**. Molecular lesions in new alleles of *Mad*, *punt* and *60A*. The position and nature of the mutations are indicated below the schematic diagram of the protein. Asterisk, nonsense mutation. (A) Mutations in new *Mad* alleles. The hatched box represents the mutation hot spot. (B) Mutations in new *punt* alleles. The structural features shown are the putative signal peptide (oval box), the extracellular cysteine residues (vertical bars), the transmembrane domain (hatched box) and the kinase domain (open box). (C) Mutations in *60A* alleles. Letter Cs within the mature domain represent conserved cysteine residues.

## Loss-of-function phenotypes of *60A*

Animals lacking *60A* function died at late larval/early pupal stages. One of the striking phenotypes of *60A* mutant larvae is a transparent appearance due to the lack of fat body (Fig. 5B). In roughly 50% of the *60A* larvae, the gastric caecae were reduced in length (Fig. 5D), consistent with the expression of *60A* in the gastric caecae (Doctor et al., 1992). These phenotypes are similar to those of the *Mad* mutant larvae (Sekelsky et al., 1995).

During embryogenesis, *60A* is expressed throughout the visceral mesoderm of the developing midgut (Doctor et al., 1992) suggesting a function for *60A* in gut development. Indeed, embryos lacking *60A* failed to form the first constriction (Fig. 6E,F). The homeotic gene *Antennapedia (Antp)* is expressed in the visceral mesoderm around the first constriction and is required for its formation (Reuter and Scott, 1990; Tremml and Bienz, 1989). We examined the *Antp* expression in *60A* mutant embryos. Consistent with the lack of the first constriction, *Antp* expression was greatly reduced (Fig. 6B). Thus, *60A* is required for the formation of the first constriction of the midgut, likely through positively regulating the expression of *Antp* in the visceral mesoderm.

## *60A* maintains an optimal level of *dpp* signaling in the visceral mesoderm

The identification of mutations in *60A* as dominant enhancers of $tkv^6$, thus *dpp* signaling, in the imaginal discs raised the possibility that *60A* is required for optimal signaling by the *dpp* pathway. To determine if there was a general requirement for *60A* in *dpp* signaling, we examined the effects of *60A* mutations on *dpp* signaling in the visceral mesoderm where both *dpp* and *60A* are expressed.

*dpp* is expressed in two discrete domains in the visceral mesoderm (Panganiban et al., 1990b). The anterior domain of *dpp* coincides with the gastric caecae primordia, which are immediately anterior to the expression domain of *Sex combs reduced (Scr)* in parasegment (ps) 4. The failure to initiate *dpp* expression in ps3 in $dpp^{shv}$ mutants results in anterior expansion of *Scr* expression and arrested outgrowth of the gastric caecae (Panganiban et al., 1990b; Hursh et al., 1993), indicating a role for *dpp* in repressing *Scr* in ps3. $tkv^6$ homozygotes are homozygous viable, so it is not surprising that all the midgut gene expression patterns examined were essentially normal (Fig. 7A-D). *Scr* expression in $tkv^6$ and *60A* mutants was normal (Fig. 7A,E). However, in $tkv^6$ and *60A* double mutants, the *Scr* expression extended anteriorly into ps3 (Fig. 7I) as it did in $dpp^{shv}$ mutants, suggesting that *60A* activity is required in ps3 for optimal *dpp* signaling.

To test whether *60A* also acts synergistically with *dpp* elsewhere in the midgut, we examined the gene expression of *dpp* and *Ultrabithorax* (*Ubx*) in ps7 and *wingless* (*wg*) in the

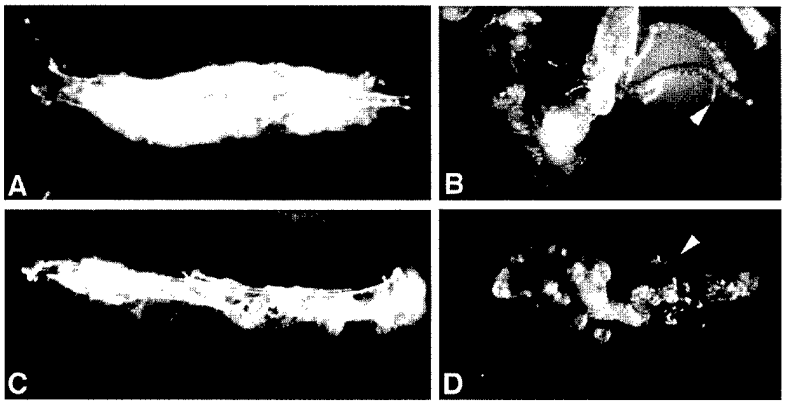



**Fig. 5.** Larval phenotypes of *60A*. (A) Wild-type third instar larva. (C) $60A^{D4}/60A^{D8}$ third instar larva had greatly reduced fat body and appeared transparent. (B) Wild-type third instar larval gut. Note the long and extended gastric caecae (arrowhead). (D) $60A^{D4}/60A^{D8}$ third instar larval gut. The gastric caecae were short in about half of the *60A* larvae (n=47) (arrowhead). Compared with the wild-type larva of the same stage, *60A* mutants grew more slowly and were somewhat reduced in size.

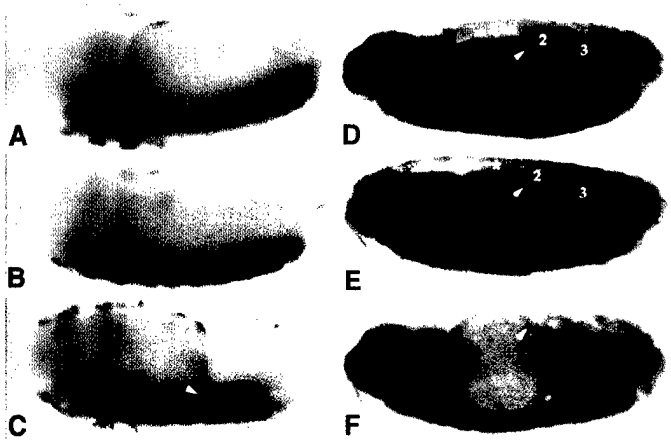



**Fig. 6.** *60A* mutants lacked the first constriction of the embryonic midgut. Lateral views of embryos stained with anti-Antp antibody (A-C) and anti-Lab antibody (D-F); stage 14 (A, B), stage 15 (C), stage 16 (D-F). Anterior is to the left and dorsal is up for this and subsequent figures. (A) Phenotypically normal visceral mesoderm expression of *Antp* in ps6 in $tkv^6$ homozygous embryos (bracket). (B) $60A^{D4}/60A^{D8}$ embryos lacked ps6 *Antp* expression (bracket). (C) $tkv^6 60A^{D4}/tkv^6 60A^{D8}$ embryos lacked *Antp* expression in ps6 (bracket) but acquired an ectopic *Antp* domain in ps7 (arrowheads) presumably due to lacking *Ubx* expression in ps7 (see Fig. 7L). (D) $tkv^6$ homozygotes had normal *lab* expression (arrowhead) in the endoderm and formed three constrictions indicated by numbers. (E) $60A^{D4}/60A^{D8}$ embryos failed to form the first constriction (asterisk) but the second and third constrictions still formed. *lab* expression was not altered (arrowhead). (F) $tkv^6 60A^{D4}/tkv^6 60A^{D8}$ embryos lacked *lab* expression (arrowhead) in the endoderm and only formed one constriction.

adjacent ps8. It has been established that ps7 expression of *dpp* is activated by the homeotic gene *Ubx* (Immerglück et al., 1990; Panganiban et al., 1990b; Reuter et al., 1990; Capovilla et al., 1994; Sun et al., 1995) and maintained by an autostimulatory circuit involving *dpp*, *Ubx* and *wg* (Hursh et al., 1993; Thüringer et al., 1993a,b; Staehling-Hampton and Hoffmann, 1994). The proper expression of all three genes is interdependent and critical for maintaining a stable cellular differentiation commitment (Bienz, 1994). The expression of *dpp* and *wg* in the visceral mesoderm is required for the induction of the homeotic gene *labial* (*lab*) in the underlying endoderm (Immerglück et al., 1990; Panganiban et al., 1990b; Reuter et al., 1990). The absence of *dpp* function in ps7 disrupts the autoregulatory loop and reduces the expression of *Ubx*, *wg*, *dpp* and *lab* in ps7, leading to the absence of the second constriction in *dpp* mutant embryos (Immerglück et al., 1990; Panganiban et al., 1990b; Reuter et al., 1990).

We found that animals mutant for either $tkv^6$ or *60A* had normal expression of *dpp*, *wg* and *Ubx* (Fig. 7B-D,F-H). However, in $tkv^6$ *60A* double mutants, *dpp* expression in ps3 and ps7 was greatly reduced (Fig. 7J). The initiation of *dpp* expression at earlier stages was not affected in the double mutants (not shown), suggesting that the reduction of *dpp* expression resulted from failure to maintain its expression at later stages. Similarly, in *Mad* mutants, the initiation of *dpp* expression in ps3 and ps7 is not affected but the maintenance of *dpp* expression does not occur (Newfeld et al., 1997). This is because *dpp* expression is activated directly by *Ubx* and only its maintenance requires positive feedback involving *dpp* signaling. In the double mutants, *Ubx* expression in ps7 and *wg* expression in ps8 were greatly reduced (Fig. 7K,L), suggesting the disruption of the positive regulatory loop. The

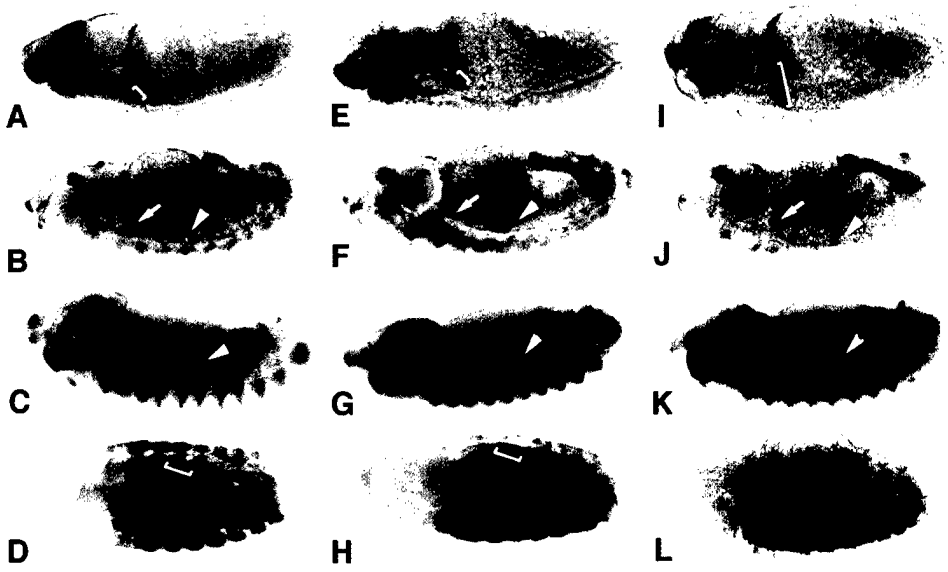



**Fig. 7.** *60A* enhanced $tkv^6$ midgut phenotypes. Lateral views of embryos stained with anti-Scr antibody (A, E, I); anti-Dpp antibody (B, F, J); anti-Wg antibody (C, G, K) and anti-Ubx antibody (D, H, L). A-D, $tkv^6$ homozygotes; E-H, $60A^{D4}/60A^{D8}$ embryos; I-L, $tkv^6 60A^{D4}/tkv^6 60A^{D8}$ embryos. C, G and K were at stage 14; all other embryos were at stage 15. *Scr* expression in ps4 was normal in $tkv^6$ (A, bracket) or *60A* mutants (E, bracket). In $tkv^6$*60A* embryos, *Scr* extended anteriorly into ps3 (I, bracket). The visceral mesoderm expression of *dpp* in the single mutants were normal (B, F, arrows, gastric caecae; arrowheads, ps7). Double mutants had greatly reduced expression of *dpp* in gastric caecae (J, arrow) and in ps7 (J, arrowhead). *dpp* expression in the other domains were unaffected. *wg* was expressed normally in the single mutants (C, G, arrowhead), however, ps8 expression is undetectable in the double mutants (K, arrowhead). *Ubx* expression in ps7 was normal in $tkv^6$ (D, bracket) or *60A* mutants (H, bracket), but was greatly reduced in the double mutants (L, bracket).

reduction of *dpp* in ps3 in the double mutants may explain the observed derepression of *Scr*.

*Ubx* is required for repressing *Antp* in ps6. In *Ubx* mutants, the *Antp* domain is extended posteriorly into ps7 (Tremml and Bienz, 1989), indicating a homeotic transformation of ps7 into ps6. A similar phenotype was observed for *tkv* null embryos (Affolter et al., 1994). Consistent with the argument that lacking *60A* compromises *dpp* signaling, there was also a posterior expansion of *Antp* in $tkv^6$*60A* double mutants (Fig. 6C). Interestingly, due to the *60A* mutation, the endogenous *Antp* expression was absent, such that there was only ectopic *Antp* in ps7, where *Ubx* would normally be.

We also examined the expression of *lab* in the endoderm. Consistent with the gene expression changes in the visceral mesoderm, *lab* expression was not affected by $tkv^6$ or *60A* mutations. However, it was greatly reduced in $tkv^6$*60A* double mutants (Fig. 6F). The gut of the double mutants only formed two chambers instead of the normal four chambers (Fig. 6G, compare to 6D). This phenotype likely resulted from the failure to form the first constriction due to lacking *60A* function and the failure to form the second constriction due to lacking *dpp* signaling. It is unclear why the position of the only constriction observed in the double mutants is somewhat more anterior than a normal third constriction.

The gene expression changes in the midgut of the $tkv^6$*60A* double mutants are consistent with *60A* playing a role in augmenting *dpp* signaling.

### *60A* enhances the ectodermal phenotypes of $tkv^6$ homozygotes

Previous studies have established *dpp*'s role as a morphogen in patterning the embryonic ectoderm (Ferguson and Anderson, 1992a,b; Wharton et al., 1993). *dpp* signaling is also required for dorsal closure of the embryonic ectoderm (Hou et al., 1997; Riesgo-Escovar and Hafen, 1997). We therefore examined if the level of *60A* affected the phenotype of the embryonic ectoderm.

We compared the cuticle phenotypes of single and double mutants. Since $tkv^6$ homozygotes were viable and *60A* mutants had no obvious defects until late in development, the cuticular patterns of these mutants were essentially normal (Fig. 8A,B). However, $tkv^6$*60A* homozygote embryos died and exhibited head defects and an excessive ventral curvature (Fig. 8C). Although the double mutant cuticles bore some resemblance to hypomorphic *dpp* mutants, they did not exhibit an obvious expansion of the ventral denticle belts (Wharton et al., 1993). The double mutant phenotype suggested, however, that, when *dpp* signaling was compromised in the embryonic ectoderm, removing *60A* activity further attenuated *dpp* signaling. We considered that the relatively mild phenotype of the double mutant embryo might reflect partial rescue by maternally contributed wild-type Tkv receptors. Indeed, a quarter of the embryos produced by mothers homozygous for $tkv^6$ and heterozygous for *60A* exhibited a dorsal open phenotype similar (Fig. 8D) to that of zygotic *tkv* null embryos (Penton et al., 1994). Therefore, in the absence of maternally provided wild-type Tkv, $tkv^6$*60A* double mutant embryos exhibit a phenotype indicative of defective *dpp* signaling during the process of dorsal closure.

## DISCUSSION

The haploinsufficiency of the *dpp* locus reflects the sensitivity of developmental processes to a reduction in *dpp* signaling. We have carried out a genetic screen to search for modifiers of $tkv^6$, a hypomorphic type I *dpp* receptor. *tkv* has been implicated in all aspects of *dpp* signaling both in vitro and in vivo (Penton et al., 1994; Nellen et al., 1994; Brummel et al., 1994; Affolter et al., 1994; Burke and Basler, 1996; Singer et al., 1997). Therefore, the modifiers of $tkv^6$ are most likely to be integral components of the *dpp* signal transduction pathway.

### Identification of new alleles of *tkv, Mad, Med, punt, 60A* and two new loci as dominant enhancers of $tkv^6$

New alleles of several genes known to mediate *dpp* signaling were identified, including *tkv*, *punt*, *Mad* and *Med*. Recovery of these mutations as dominant enhancers of $tkv^6$ validated the specificity of the screen.

We isolated five alleles of *Mad*, a key signal transducer in *dpp* signaling (Newfeld et al., 1996; Kim et al., 1997). Three of them have point mutations in the coding region (Fig. 4A). The molecular lesions correlate with their phenotypic properties. $Mad^{D14}$ has a nonsense mutation predicted to produce a truncated protein with only the conserved MH1 domain. It behaves genetically as a null. $Mad^{D16}$ has a tyrosine-to-asparagine change in the divergent linker region and behaves as a hypomorph with residual activity (Table 2). This suggests that the amino acid change only partially affects the protein function. The MH2 domains of Smads are highly conserved.

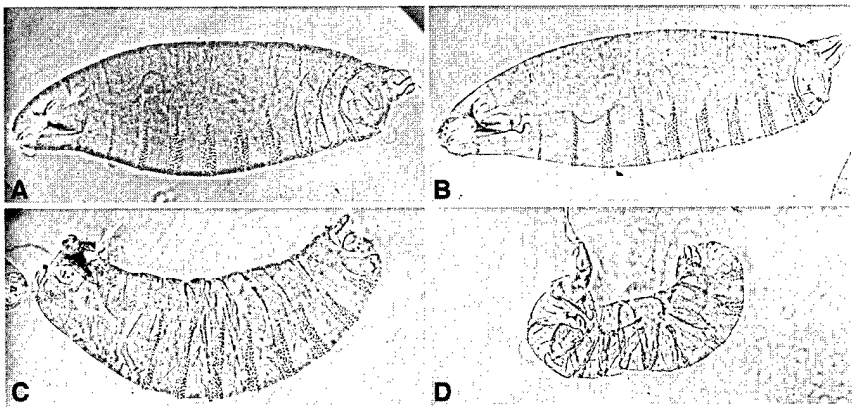



**Fig. 8.** *60A* enhanced the $tkv^6$ cuticle phenotypes. Phenotypically normal $tkv^6$ (A) and $60A^{D4}/60A^{D8}$ (B) cuticle. Note the fully internalized head skeleton (arrow). (C) $tkv^6 60A^{D4}/tkv^6 60A^{D8}$ cuticle. The partially deleted head skeletons remained external (arrow). (D) An embryo produced by $tkv^6 60A^{D4}/tkv^6$ females mated to $tkv^6 60A^{D8}/tkv^6$ males. Roughly a quarter of the embryos lacked dorsal hypoderm (arrow).

The three-dimensional structure of the MH2 domain of Smad4 indicates that Smads form homotrimers whose intact conformation is essential for the assembly of a hexamer with a different Smad homotrimer in response to receptor activation (Hata et al., 1997; Shi et al., 1997). Many of the Smad mutations associated with tumors or affecting development map to the MH2 domain. Based on the crystal structure of the carboxy domain of Smad4 (Shi et al., 1997), the invariant aspartic acid mutated to asparagine in $Mad^{D24}$ maps to the trimer interface region critical for trimerization. The corresponding residue in Smad2 is mutated in colon cancers (Eppert et al., 1996). Interestingly, the $Mad^{D24}$ mutation resulted in dominant female sterility (Y. C. and F. M. H., unpublished data) which is not observed with *Mad* null alleles, suggesting that it has a dominant negative effect, perhaps by forming inactive oligomers with the wild-type proteins in the heterozygotes.

Analysis of the two new *punt* alleles also provides evidence for the in vivo importance of conserved structural motifs in this type II *dpp* receptor (Fig. 4B). $punt^{D13}$ has a cysteine-to-tyrosine change in the extracellular cysteine cluster characteristic of all receptors for TGF-β superfamily members (Massagué et al., 1994). $punt^{D18}$ changes the highly conserved glutamic acid to a valine in the catalytic core of the kinase domain, where another punt mutation, $punt^{135}$, is mapped (Ruberte et al., 1995). Like $punt^{135}$, both new *punt* alleles display some temperature sensitivity (Y. C. and F. M. H., unpublished data), suggesting that they are not protein nulls. No null mutations in the *punt* locus have been reported, suggesting that like *dpp*, *punt* may be haploinsufficient.

We did not isolate any new alleles of *shn*, which enhanced $tkv^6$ in the initial test. The enhancement by $shn^{IB}$ may be allele specific, such that a particular form of mutant Shn protein is needed to produce an enhancement. Consistent with this, $shn^p$, which makes no detectable protein (Staehling-Hampton et al., 1995), failed to enhance the $tkv^6$ phenotype (data not shown). No *dpp* alleles were recovered either, possibly due to the haploinsufficiency associated with the locus and the fact that most hypomorphic *dpp* mutations affect regulatory regions, which are less likely to be affected by chemical mutagens such as ENU.

One unexpected locus identified in our screen is *60A*, which encodes the BMP-7 homologue (Doctor et al., 1992; Wharton et al., 1991). The fact that in a screen of the entire genome, *60A* mutations were recovered multiple times as dominant enhancers of a mutant *dpp* receptor provides strong evidence for its involvement in *dpp* signaling. Nonsense mutations were found in all three alleles of *60A* in the prodomain of the precursor protein. Since these mutations are predicted to eliminate translation of the biologically active mature C-terminal domain, they most likely represent functional nulls of the *60A* gene.

## The developmental functions of *60A* and its role in *dpp* signaling

Phenotypic analysis of *60A* single mutants and $tkv^6$*60A* double mutants revealed both *dpp*-independent and *dpp*-dependent functions for *60A*. *60A* is expressed broadly throughout development, with enrichment in the developing gut (Doctor et al., 1992), suggesting a role for *60A* in gut morphogenesis. *60A* mutants lack the first constriction of the embryonic midgut and *Antp* expression in ps6, indicating that *60A* is required for the formation of the first constriction, possibly through regulating *Antp* expression. This function is independent of *dpp* signaling, since mutations in *dpp* or its receptors only disrupt the formation of the second but not the first constriction (Panganiban et al., 1990b; Nellen et al., 1994; Ruberte et al., 1995). This also suggests that there is either redundancy or that a different receptor system is responsible for mediating *60A* signaling to pattern the first constriction. It would be interesting to see if AtrI (Childs et al., 1993), a type I receptor and STK-D (Ruberte et al., 1995), a type II receptor in *Drosophila*, both of unknown function, are mediators of *60A* signaling at the site of the first constriction.

The fact that *60A* mutations are dominant enhancers of a sensitized *dpp* pathway implicates *60A* in potentiating *dpp* signaling. This is most obvious in the visceral mesoderm of the midgut where *dpp* signaling is required to regulate homeotic gene expression and to maintain its own expression through a positive feedback mechanism. Although *dpp* signaling in the visceral mesoderm appears intact in *60A* mutants, a requirement for *60A* is revealed in $tkv^6$*60A* double mutants. When *dpp* signaling is attenuated through a mutant *tkv* receptor, eliminating *60A* function reduces the signaling to below threshold level. The derepression of *Scr* in the anterior midgut and the loss of expression of *dpp* target genes, *wg*, *Ubx* and *dpp*, in the visceral mesoderm and *lab* in the endoderm are consistent with inadequate *dpp* signaling. A similar requirement for *60A* is observed during dorsal closure of the embryonic ectoderm. The enhanced phenotypes of the adult appendages closely resemble those of the *dpp* hypomorphic mutants (Spencer et al., 1982), suggesting that *60A* activity is also required for imaginal disc patterning. It is interesting that the imaginal discs are more sensitive to the reduction of *60A* function, as a 50% reduction in *60A* function is sufficient to produce a phenotype in a $tkv^6$ genetic background. This may reflect a differential threshold requirement for *dpp* signaling in different tissues. Taken together, our data argue for an involvement of *60A* in *dpp* signaling at different developmental stages and in various tissues.

In a signaling system with multiple interacting dimeric ligands, the interpretation of any single mutant phenotypes must consider the effect of losing both homomeric and possible heteromeric ligands. Therefore, the functions of the *dpp* pathway may be a composite input from Dpp homodimers, and Dpp/Scw and Dpp/60A heterodimers. Alternatively, 60A homodimers may function in an additive fashion with Dpp homodimers at sites of overlapping expression. However, the loss-of-function phenotypes of *dpp* are as severe as the loss-of-function phenotypes of its downstream components, such as *tkv* or *Mad* (Padgett et al., 1987; Penton et al., 1994; Nellen et al., 1994; Newfeld et al., 1996, 1997), suggesting that there is very little signaling, if any at all, from 60A homodimers in *dpp*-dependent events. Therefore, we believe it is unlikely that 60A homodimers play a significant role in *dpp*-dependent processes. Rather, we favor the interpretation that Dpp/60A heterodimers form at sites of overlapping expression and participate with Dpp homodimers in multiple signaling events.

The overlapping expression patterns of murine BMPs have led to the suggestion that they may act combinatorially during development (Lyons et al., 1995). Given the dimeric nature of TGF-β superfamily ligands, one mechanism to achieve such a

combinatorial effect is to form functional heterodimers. Heterodimers of *Xenopus* BMP-4 and BMP-7 have been generated in vitro (Hazama et al., 1995) and shown to be more potent in bone- (Aono et al., 1995) and mesoderm-inducing assays (Suzuki et al., 1997) than either homodimer. In *Drosophila*, the Scw protein is proposed to upregulate *dpp* activity by forming Dpp/Scw heterodimers in the dorsal/ventral patterning of the embryonic ectoderm (Arora et al., 1994). The broad distribution of 60A proteins provides an opportunity for forming Dpp/60A heterodimers. Unlike *scw* null mutations, no obvious disruption of *dpp* signaling is observed in *60A* null mutants, suggesting that Dpp/60A heterodimers are not as limiting as Dpp/Scw heterodimers, but partially redundant with Dpp homodimers. The first constriction phenotype of *60A* mutants is unique, suggesting that it may be a function of 60A homodimers.

The $tkv^6$ receptor failed to bind homomeric ligands when expressed in COS cells (Penton et al., 1994). Since *dpp* signaling is intact in $tkv^6$ mutants, it would be interesting to determine if the mutant receptor still binds Dpp/60A and/or Dpp/scw heterodimers. If this is the case, the severe midgut phenotypes of $tkv^6$*60A* double mutants could be due to additional loss of signaling from Dpp/60A heterodimers, which would be consistent with the proposal that *60A* potentiates *dpp* signaling by forming heterodimers with Dpp. The cuticular phenotypes of $tkv^6$*60A* double mutants without maternal wild-type Tkv receptor do not exhibit the altered dorsal/ventral polarity observed in *dpp* null embryos, possibly due to Dpp/Scw heterodimer signaling through the $tkv^6$ receptor during early embryogenesis.

In summary, we have isolated mutant alleles of genes involved in *dpp* signaling, including *60A*. Mutations in *60A* disrupt *dpp* signaling in multiple developmental processes when *dpp* signaling is compromised. We propose that *60A* participates in *dpp* signaling by forming heterodimers with Dpp protein. Our data support both *dpp*-dependent and *dpp*-independent functions for *60A*. It remains to be determined if these distinct functions reflect the qualitative differences between different forms of the ligands and if they are mediated by differentially activated receptors or distinct cytoplasmic signal transducers. The availability of *60A* mutations provides a genetic tool for dissecting the differential requirement of each component in this combinatorial signaling system.

We thank S. Ahern-Djamali for critical reading of the manuscript. We also thank other members of the Hoffmann laboratory for helping with the screen. Y. C. thanks H. Zhang for his support. This work was supported by Department of the Army grant DAMD17-94-J-4339 to F. M. H. and by Cancer Center Core support CA07175 to the McArdle Laboratory. F. M. H. is the recipient of a Faculty Research Award from the American Cancer Society.

## REFERENCES

**Affolter, M., Nellen, D., Nussbaumer, U. and Basler, K.** (1994). Multiple requirements for the receptor serine/threonine kinase *thick veins* reveal novel functions of TGF-β homologs during *Drosophila* embryogenesis. *Development* **120,** 3105-3117.

**Aono, A., Hazama, M., Notoya, K., Taketomi, S., Yamasaki, H., Tsukuda, R., Sasaki, S. and Fujisawa, Y.** (1995). Potent ectopic bone-inducing activity of bone morphogenetic protein-4/7 heterodimer. *Biochem. Biophys. Res. Commun.* **210,** 670-677.

**Arora, K., Dai, H., Kazuko, S. G., Jamal, J., O'Connor, M. B., Letsou, A. and Warrior, R.** (1995). The *Drosophila schnurri* gene acts in the Dpp/TGF-β signaling pathway and encodes a transcription factor homologous to the human MBP family. *Cell* **81,** 781-790.

**Arora, K., Levine, M. S. and O'Connor, M. B.** (1994). The *screw* gene encodes a ubiquitously expressed member of the TGF-β family required for specification of dorsal cell fates in the *Drosophila* embryo. *Genes Dev.* **8,** 2588-2601.

**Ashburner, M.** (1989). *Drosophila: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

**Bienz, M.** (1994). Homeotic genes and positional signalling in the *Drosophila* viscera. *Trends Genet.* **10,** 22-26.

**Brook, W. J., Diaz-Benjumea, F. J. and Cohen, M. S.** (1996). Organizing spatial pattern in limb development. *Annu. Rev. Cell Dev. Biol.* **12,** 161-180.

**Brummel, T. J., Twombly, V., Marqué, G., Wrana, J. L., Newfeld, S. J., Attisano, L., Massagué, J., O'Connor, M. B. and Gelbart, W. M.** (1994). Characterization and relationship of Dpp receptors encoded by the *saxophone* and *thick veins* genes in *Drosophila*. *Cell* **78,** 251-261.

**Burke, R. and Basler, K.** (1996). Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. *Development* **122,** 2261-2269.

**Capovilla, M., Brandt, M. and Botas, J.** (1994). Direct regulation of *decapentaplegic* by *Ultrabithorax* and its role in *Drosophila* midgut morphogenesis. *Cell* **76,** 461-475.

**Childs, S. R., Wrana, J. L., Arora, K., Attisano, L., O'Connor, M. B. and Massagué, J.** (1993). Identification of a *Drosophila* activin receptor. *Proc. Natl. Acad. Sci. USA* **90,** 9475-9479.

**Derynck, R. and Zhang, Y.** (1996). Intracellular signalling: the mad way to do it. *Curr. Biol.* **6,** 1226-1229.

**Doctor, J. S., Jackson, P. D., Rashka, K. E., Visalli, M. and Hoffmann, F. M.** (1992). Sequence, biochemical characterization, and developmental expression of a new member of the TGF-β superfamily in *Drosophila melanogaster*. *Dev. Biol.* **151,** 491-505.

**Eppert, K., Scherer, S. W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L. C., Bapat, B., Gallinger, S., Andrulis, I. L., Thomsen, G. H., Wrana, J. L. and Attisano, L.** (1996). MADR2 maps to 18q21 and encodes a TGFβ-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* **86,** 543-552.

**Ferguson, E. L. and Anderson, K. V.** (1992a). *decapentaplegic* acts as a morphogen to organize dorsal-ventral pattern in the *Drosophila* embryo. *Cell* **71,** 451-461.

**Ferguson, E. L. and Anderson, K. V.** (1992b). Localized enhancement and repression of the activity of the TGF-β family member, *decapentaplegic*, is necessary for dorsal-ventral pattern formation in the *Drosophila* embryo. *Development* **114,** 583-597.

**Gertler, F. B., Doctor, J. S. and Hoffmann, F. M.** (1990). Genetic suppression of mutations in the *Drosophila abl* Proto-oncogene homolog. *Science* **248,** 857-860.

**Grieder, N. C., Nellen, D., Burke, R., Basler, K. and Affolter, M.** (1995). *schnurri* is required for *Drosophila dpp* signaling and encodes a zinc finger protein similar to the mammalian transcription factor PRDII-BF1. *Cell* **81,** 791-800.

**Hata, A., Lo, R. S., Wotton, D., Lagna, G. and Massagué, J.** (1997). Mutations increasing autoinhibition inactivate tumour suppressors Samd2 and Smad4. *Nature* **388,** 82-87.

**Hazama, M., Aono, A., Ueno, N. and Fujisawa, Y.** (1995). Efficient expression of a heterodimer of bone morphogenetic protein subunits using a baculovirus expression system. *Biochem. Biophys. Res. Commun.* **209,** 859-866.

**Hogan, B. L.** (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10,** 1580-1594.

**Hou, X.S., Goldstein, E.S. and Perrimon, N.** (1997). Drosophila Jun relays the Jun amino-terminal kinase signal transduction pathway to the Decapentaplegic signal transduction pathway in regulating epithelial cell sheet movement. *Genes Dev.* **11,** 1728-1737.

**Hursh, D. A., Padgett, R. W. and Gelbart, W. M.** (1993). Cross regulation of *decapentaplegic* and *Ultrabithorax* transcription in the embryonic visceral mesoderm of *Drosophila*. *Development* **117,** 1211-1222.

**Immerglück, K., Lawrence, P. A. and Bienz, M.** (1990). Induction across germ layers in *Drosophila* mediated by a genetic cascade. *Cell* **62,** 261-268.

**Jackson, P. D. and Hoffmann, F. M.** (1994). Embryonic expression patterns of the *Drosophila decapentaplegic* gene: separate regulatory elements control blastoderm expression and lateral ectodermal expression. *Developmental Dynamics* **199,** 28-44.

**Kim, J., Johnson, K., Chen, H. J., Carroll, S. and Laughon, A.** (1997). *Drosophila* Mad binds to DNA and directly mediates activation of *vestigial* by Decapentaplegic. *Nature* **388,** 304-308.

**Kingsley, D. M.** (1994a). The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.* **8,** 133-146.

**Kingsley, D. M.** (1994b). What do BMPs do in mammals? Clues from the mouse *short-ear* mutation. *Trends Genet.* **10,** 16-21.

**Letsou, A., Arora, K., Wrana, J. L., Simin, K., Twombly, V., Jamal, J., Staehling-Hampton, K., Hoffmann, F. M., Gelbart, W. M., Massagué, J. and et al.** (1995). *Drosophila* Dpp signaling is mediated by the *punt* gene product: a dual ligand-binding type II receptor of the TGFβ receptor family. *Cell* **80,** 899-908.

**Lyons, K. M., Hogan, B. L. and Robertson, E. J.** (1995). Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development. *Mech. Dev.* **50,** 71-83.

**Massagué, J.** (1996). TGF-β signaling: receptors, transducers, and Mad proteins. *Cell* **85,** 947-950.

**Massagué, J., Attisano, L. and Wrana, J. L.** (1994). The TGF-β family and its composite receptors. *Trends Cell Biol.* **4,** 172-178.

**Massagué, J. and Weis-Garcia, F.** (1996). Serine/threonine kinase receptors: mediators of transforming growth factor β family signals. *Cancer Surv.* **27,** 41-64.

**Masucci, J. D. and Hoffmann, F. M.** (1993). Identification of two regions from the *Drosophila decapentaplegic* gene required for embryonic midgut development and larval viability. *Dev. Biol.* **159,** 276-287.

**Masucci, J. D., Miltenberger, R. J. and Hoffmann, F. M.** (1990). Pattern-specific expression of the *Drosophila decapentaplegic* gene in imaginal disks is regulated by 3′ cis-regulatory elements. *Genes Dev.* **4,** 2011-2023.

**Miyazono, K., Ichijo, H. and C-H., H.** (1993). Transforming growth factor-β: latent forms, binding proteins and receptors. *Growth Factors* **8,** 11-22.

**Nellen, D., Affolter, M. and Basler, K.** (1994). Receptor serine/threonine kinases implicated in the control of *Drosophila* body pattern by *decapentaplegic*. *Cell* **78,** 225-237.

**Newfeld, S. J., Chartoff, E. H., Graff, J. M., Melton, D. A. and Gelbart, W. M.** (1996). *Mothers against dpp* encodes a conserved cytoplasmic protein required in DPP/TGF-β responsive cells. *Development* **122,** 2099-2108.

**Newfeld, S. J., Mehra, A., Singer, M. A., Wrana, J. L., Attisano, L. and Gelbart, W. M.** (1997). *Mothers against dpp* participates in a DPP/TGF-β responsive serine-threonine kinase signal transduction cascade. *Development* **124,** 3167-3176.

**Padgett, R. W., St. Johnston, R. D. and Gelbart, W. M.** (1987). A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-β family. *Nature* 81-84.

**Padgett, R. W., Wozney, J. M. and Gelbart, W. M.** (1993). Human BMP sequences can confer normal dorsal-ventral patterning in the *Drosophila* embryo. *Proc. Natl. Acad. Sci. USA* **90,** 2905-2909.

**Panganiban, G. E. F., Rashka, K. E., Neitzel, M. D. and Hoffmann, F. M.** (1990a). Biochemical characterization of the *Drosophila* Dpp protein, a member of the transforming growth factor-β family of growth factors. *Mol. Cell. Biol.* **10,** 2669-2677.

**Panganiban, G. E. F., Reuter, R., Scott, M. P. and Hoffmann, F. M.** (1990b). A *Drosophila* growth factor homolog, *decapentaplegic*, regulates homeotic gene expression within and across germ layers during midgut morphogenesis. *Development* **110,** 1041-1050.

**Penton, A., Chen, Y., Staehling-Hampton, K., Wrana, J. L., Attisano, L., Szidonya, J., Cassill, J. A., Massagué, J. and Hoffmann, F. M.** (1994). Identification of two bone morphogenetic protein type I receptors in *Drosophila* and evidence that Brk25D is a *decapentaplegic* receptor. *Cell* **78,** 239-250.

**Raftery, L. A., Twombly, V., Wharton, K. and Gelbart, W. M.** (1995). Genetic screens to identify elements of the *decapentaplegic* signaling pathway in *Drosophila*. *Genetics* **139,** 241-254.

**Riesgo-Escovar, J.R. and Hafen, E.** (1997). *Drosophila* Jun kinase regulates expression of *decapentaplegic* via the ETS-domain protein Aop and the AP-1 transcription factor *DJun* during dorsal closure. *Genes Dev.* **11,** 1717-1727.

**Reuter, R., Panganiban, G. E. F., Hoffmann, F. M. and Scott, M. P.** (1990). Homeotic genes regulate the spatial expression of putative growth factors in the visceral mesoderm of *Drosophila* embryos. *Development* **110,** 1031-1040.

**Reuter, R. and Scott, M. P.** (1990). Expression and function of the homeotic genes *Antennapedia* and *Sex combs reduced* in the embryonic midgut of *Drosophila*. *Development* **109,** 289-303.

**Roberts, A. B. and Sporn, M. B.** (1990). The transforming growth factor-betas. In *Peptide growth factors and their receptors*, (ed. M. B. Sporn and A. B. Roberts), pp. 419-472. Springer-Verlag, Heidelberg

**Ruberte, E., Narty, T., Nellen, D., Affolter, M. and Basler, K.** (1995). An absolute requirement for both the type II and type I receptors, *Punt* and *thickveins*, for *dpp* signaling *in vivo*. *Cell* **80,** 889-897.

**Sampath, T. K., Rashka, K. E., Doctor, J. S., Tucker, R. F. and Hoffmann, F. M.** (1993). *Drosophila* TGF-β superfamily proteins induce endochondral bone formation in mammals. *Proc. Natl. Acad. Sci USA* **90,** 6004-6008.

**Sekelsky, J., Newfeld, S. J., Raftery, L. A., Chartoff, E. H. and Gelbart, W. M.** (1995). Genetic characterization and cloning of *mothers against dpp*, a gene required for *decapentaplegic* function in *Drosophila melanogaster*. *Genetics* **139,** 1347-1358.

**Shi, Y., Hata, A., Lo, R. S., Massagué, J. and Pavletich, N. P.** (1997). A structural basis for mutational inactivation of the tumour suppressor Smad4. *Nature* **388,** 87-93.

**Simon, M. A., Bowtell, D. D. L., Dodson, G. S., Laverty, T. R. and Rubin, G. M.** (1991). Ras 1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the Sevenless protein tyrosine kinase. *Cell* **67,** 701-716.

**Singer, M. A., Penton, A., Twombly, V., Hoffmann, F. M. and Gelbart, W. M.** (1997). Signaling through both type I DPP receptors is required for anterior-posterior patterning of the entire *Drosophila* wing. *Development* **124,** 79-89.

**Spencer, F. A., Hoffmann, F. M. and Gelbart, W. M.** (1982). *Decapentaplegic*: A gene complex affecting morphogenesis in *Drosophila melanogaster*. *Cell* **28,** 451-461.

**St. Johnston, R. D. and Gelbart, W. D.** (1987). *Decapentaplegic* transcripts are localized along the dorsal-ventral axis of the *Drosophila* embryo. *EMBO J.* **6,** 2785-2791.

**Staehling-Hampton, K. and Hoffmann, F. M.** (1994). Ectopic *decapentaplegic* in the *Drosophila* midgut alters the expression of five homeotic genes, *dpp* and *wingless*, causing morphological defects. *Dev. Biol.* **164,** 502-512.

**Staehling-Hampton, K., Hoffmann, F. M., Baylies, M. K., Rushton, E. and Bate, M.** (1994). *dpp* induces mesodermal gene expression in *Drosophila*. *Nature* **372,** 783-786.

**Staehling-Hampton, K., Laughon, A. S. and Hoffmann, F. M.** (1995). A *Drosophila* protein related to the human zinc finger transcription factor PRDII/MBPI/HIV-EP1 is required for *dpp* signaling. *Development* **121,** 3393-3403.

**Struhl, G.** (1989). Differing strategies for organizing anterior and posterior body pattern in *Drosophila* embryos. *Nature* **338,** 741-744.

**Sun, B., Hursh, D. A., Jackson, D. and Beachy, P. A.** (1995). *Ultrabithorax* protein is necessary but not sufficient for full activation of *decapentaplegic* expression in the visceral mesoderm. *EMBO J.* **14,** 520-535.

**Suzuki, A., Kaneko, E., Maeda, J. and Ueno, N.** (1997). Mesoderm induction by BMP-4 and -7 heterodimers. *Biochem. Biophys. Res. Commun.* **232,** 153-156.

**Thüringer, F., Cohen, S. M. and Bienz, M.** (1993a). Dissection of an indirect autoregulatory response of a homeotic *Drosophila* gene. *EMBO J.* **12,** 2419-2430.

**Thüringer, F., Cohen, S. M. and Bienz, M.** (1993b). Indirect autoregulation of a homeotic *Drosophila* gene mediated by extracellular signaling. *Proc. Natl. Acad. Sci. USA* **90,** 3899-3903.

**Tremml, G. and Bienz, M.** (1989). Homeotic gene expression in the visceral mesoderm of *Drosophila* embryos. *EMBO J.* **8,** 2677-2685.

**Twombly, V., Blackman, R. K., Jin, H., Graff, J. M., Padgett, R. W. and Gelbart, W. M.** (1996). The TGF-β signaling pathway is essential for *Drosophila* oogenesis. *Development* **122,** 1555-1565.

**Wharton, K. A., Ray, R. P. and Gelbart, W. M.** (1993). An activity gradient of *decapentaplegic* is necessary for the specification of dorsal pattern elements in the *Drosophila* embryo. *Development* **117,** 807-822.

**Wharton, K. A., Thomsen, G. H. and Gelbart, W. M.** (1991). *Drosophila* 60A gene, a new TGF-ß family member is closely related to human bone morphogenetic proteins. *Proc. Natl. Acad. Sci. USA* **88,** 9214-9218.

**Wrana, J. L. and Pawson, T.** (1997). Mad about SMADs. *Nature* **388,** 28-29.

**Xie, T., Finelli, A. L. and Padgett, R. W.** (1994). The *Drosophila saxophone* gene: a serine-threonine kinase receptor of the TGF-β superfamily. *Science* **263,** 1756-1759.

**Final Report for Award Number DAMD17-94-J-4339**

Publications from the Grant

Chen, Y., M.J. Riese, M.A. Killinger and F.M. Hoffmann. 1998. A genetic screen for modifiers of Drosophila *decapentaplegic* signaling identifies mutations in *punt*, *Mothers against dpp* and the BMP-7 homologue, *60A*. Development 125: 1759-1768.

List of All Personnel Receiving Pay from Grant

F. Michael Hoffmann

Yijing Chen

Peter Merriman

Mark Killinger

Shigeru Morimura

Karen Staehling-Hampton

Hui Ju Chen

Allen Comer